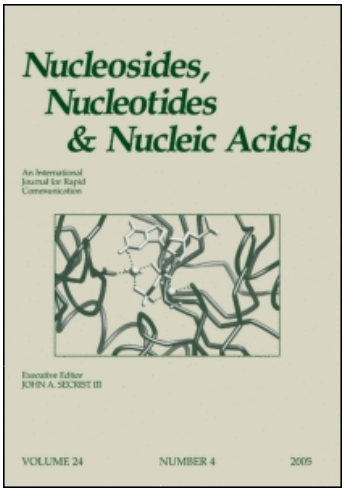

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A Novel Versatile Phosphoramidite Building Block for the Synthesis of 5'- and 3'-Hydrazide Modified Oligonucleotides

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A NOVEL VERSATILE PHOSPHORAMIDITE BUILDING BLOCK FOR THE SYNTHESIS OF 5′- AND 3′-HYDRAZIDE MODIFIED OLIGONUCLEOTIDES

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▫ *We introduce a novel versatile phosphoramidite building block for the modification of oligonucleotides (ONs) with acyl hydrazides on the 5′- or 3′-terminus, or both. The reaction of these hydrazide functionalized ONs with 4-methoxyphenylaldehyde is demonstrated for solution derivatization. Hydrazides are considered nowadays as promising reactants, which show enhanced reactivity at neutral and slightly acidic conditions and higher stability of yielding products as compared to the aliphatic amines, which are broadly used for ONs derivatization.*

Our method to introduce hydrazides into ONs employs a phosphoramidite modifier designed to split, during ammonia or lithium hydroxide treatment, into two hydrazides via β-elimination of a central bis-2-carbonylethoxysulfone unit. It allows the creation of ONs derivatized with a hydrazide moiety at the 5′-, 3′- and both 5′- and 3′-termini, as well as two different hydrazide containing ONs at the same time, viz. in one sequence on the same solid support. In latter case one can, for example, synthesize two hydrazide containing ONs, where one is 5′-modified and second one is 3′-modified.

Keywords Hydrazide, Aldehyde, Modified Oligonucleotide, Phosphoramidite Modifier, Automatic Oligonucleotide Synthesis

INTRODUCTION

The modification of ONs with nucleophilic functional groups is a routine operation nowadays enabling numerous applications and assays in molecular biology, biochemistry, and biotechnology.[1–4] One of them is amino group. In our

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opinion, hydrazide modified ONs can be considered as a rather advantageous and promising alternative to alkyl amino–modified ONs.

Briefly, it is well known that alkyl amino modified ONs react with aldehydes to produce rather unstable Schiff's bases, which require reduction to yield more stable amine derivatives.[1,5–9] Moreover, for a large variety of applications the applicability of aliphatic amino groups is limited due to the fact that their nucleophilicity heavily depends on the pH. At low pH values amines are protonated, and thus reactions with electophiles are suppressed. At high pH values amines are reactive, but in many cases basic pHs are unacceptable due to other reasons. For example, active esters, which are often used for conjugation with amines, are rather unstable at basic conditions due to quick hydrolysis. Water-soluble carbodiimides (CDIs), which are often used for conjugation of ONs with various electrophilic ligands, are readily hydrolyzed under basic conditions too. Moreover, at basic pH, CDIs may be also attacked by exocyclic amino groups of heterocyclic bases, thus producing undesirable side products.[10] Another disadvantage is acyl migration, taking place on the CDI-activated intermediate. This side process is common in CDI mediated couplings if carried out in aqueous solution.[10]

On the contrary, the reactivity of hydrazides is similar to that of amines but due to the lower pK_a values (~2.5–5 for hydrazides versus 10–11 for amines) caused by the α-effect of an NH group next to a NH_2 group in the hydrazide functionality,[11] hydrazides remain reactive at neutral and even at gently acidic conditions. The main targets for hydrazides are aldehyde functions. Reactions of hydrazides with aldehydes usually proceed rather fast, with high yield and with almost no side products. The rate constant for the reaction with aromatic aldehydes at pH 5–6 is in the order of 0.1–1 M^{-1} s^{-1} according to our experiences. Moreover, aldehydes being attacked by hydrazides form hydrazone structures which are much more stable as compared to Schiff's bases. Consequently, aldehyde containing molecules can be coupled to hydrazide functionalized ones without further reduction.[1,2,10,12,13] For example, when a 3′-terminal ribose is oxidized by sodium periodate to give a bis-aldehyde derivative, the reaction with hydrazides yields a stable dihydroxymorpholine linkage.[2,12] The hydrazone obtainment in all cases does not require any additional activation and can be readily performed in mild conditions suitable both for ONs and also double strand nucleic acids survival.

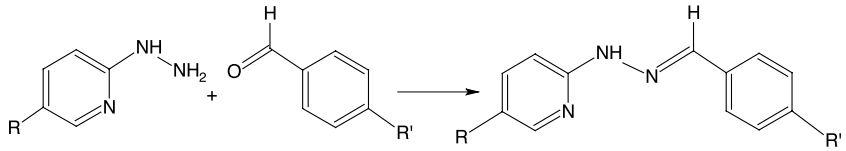
The hydrazone conjugation chemistry enables two principle approaches because the ONs can be derivatized both with hydrazide or with aldehyde groups. The advantage of a hydrazide-modified ON is that no intra- or intermolecular reaction can occur during the storage in a stock solution. This is not the case for an aldehyde-modified ON, where an intra- or intermolecular attack of an exocyclic amino group on aldehyde functionality may take place. Nevertheless, aldehyde-modified ONs are also successfully employed; for example, an immobilization of such ONs was reported on solid surfaces.[11,14]

Due to the above features, hydrazone formation has found many applications in protein and carbohydrate chemistry,[1,15] analytical biochemistry,[16–18] antisense biotechnology[19] and biochip manufacturing.[2] One group of applications deals with immobilization reactions. Examples include the immunochemical detection of glycosphingolipids on thin-layer chromatograms,[16,18] the detection of glycoproteins,[16,17] and affinity chromatography.[20,21] Synthetic ONs modified at their 5′-end with an aldehyde group can be easily attached to latex microspheres containing hydrazide moieties.[11] The coupling can be carried out under mild conditions, the reaction is usually rather fast, yielding a high binding capacity.[20] Another example of the immobilization process consists of hydrazone formation, when a 5′-aldehyde derivatized ON was immobilized on large-pore hydrazide-functionalized Sephacryl beads and this construct was used for the affinity purification of plasmid DNA via triple-helix formation.[14] Again, the strategy consists of the immobilization of ON containing electrophilic group on the 5′-end onto a support containing hydrazide group. High coupling yield and little leakage of ON material were reported.

The hydrazone chemistry has proved to be a promising way to produce oligodeoxyribonucleotide (ODN) microchips.[2,22–26] A high and reproducible immobilization yield was reported.[2] The polyacrylamide gel of chip microcells was activated by introduction of hydrazide groups, and ODNs were synthesized with a 3′-terminal 3-methyluridine unit that was then oxidized by sodium periodate to produce dialdehyde groups on the 3′-terminus.[22,23] Immobilization on hydrazide support showed high efficiency (82–93%) and low nonspecific binding (~1%).[2,24] With this technology it was reported to be possible to achieve very high immobilization capacity,[22] two orders of magnitude higher than an average two-dimensional one of a glass surface.[23]

It should nevertheless be mentioned that the hydrazone linkage was reported not to provide sufficient stability of ODN attachment onto microchips for the case when multiple hybridizations are required.[25] In these cases reductive amination is suspected to be advantageous. A number of reductants may be employed here: sodium borohydride, sodium cyanoborohydride, pyridine-borane complex, trimethylamine-borane complex, and others.[2] But even without reduction 10–15 (up to 50.[22]) cycles of hybridization could be performed in the gel microchip with reliable data output.[25,26] This is nevertheless in contrast to an older report where reduction of acyl hydrazones immobilized on latex beads was found to be mandatory.[11] Here the attachment yield of 5′-aldehyde derivatized ONs into hydrazide latex beads is roughly 50 times higher when the immobilization takes place in the presence of sodium cyanoborohydride. There are a few other examples for the immobilization of ONs on supports with hydrazone chemistry, where higher yields were reported in the presence of sodium cyanoborohydride.[20] So it is suspected, that in some cases it may be of advantage to reduce the hydrazone bond.

Nevertheless, despite the fact that a few researchers prefer to use two-step conjugation procedures that include a reduction step, in principle the hydrazone linkage is sufficiently stable under the conditions suitable for the most applications.



SCHEME 1 The formation of a stable hydrazone bond. R, R′ are the residues of molecules (e.g., peptides, proteins, fluorophores, oligonucleotides, DNA) or solid surfaces labeled with hydrazine and aldehyde moieties, respectively.

Of course, the stability of the hydrazone linkage depends on its structure. For example *TriLink Biotechnologies* offers aromatic aldehyde and aromatic hydrazine modifiers for coupling of biomolecules that are able to form a very stable hydrazone bond (Scheme 1) and does not require any further reduction to be stable.

In our opinion, even considering the possible profit of a reduction step, the hydrazone chemistry has a number of evident advantages, as it uses exceedingly mild reagents and there are virtually no competing side reactions. The only side process reported consisted of a reaction of the aldehyde group with the heterocyclic bases of DNA,[10] but it proceeds slower than the reaction of the more nucleophilic hydrazide moiety. Hydrazone adducts are usually formed with moderate yield (>80%) and reaction times vary from several minutes to several hours. Thus the hydrazone formation (sometimes followed by reduction) looks more advantageous than the coupling of aldehydes and amino derivatives which requires reductive amination in all cases.

Next to immobilization, hydrazone formation-based methods are widely used for conjugating ONs and various reporter molecules,[4,10,12,19,27,28] including markers, dyes, and peptides. Most often 5′-modified ONs are obtained through post-synthetic introduction of the hydrazide function, and they were reported to be stable in solution. Examples include methods for the direct attachment of ON probes to enzyme reporter systems.[4] High conjugation yields were reported, typically 80–85% with 3.5-fold excess of the aldehyde-modified enzyme. One more promising application of hydrazone chemistry is the possibility to create carbazoyl internucleotide linkage.[19]

There is a wealth of data about the reaction of hydrazides with bis-aldehyde derivatives of nucleosides, nucleotides and ONs yielding dihydroxymorpholines as a main product.[10,12] Hydrazide-based reagents can even be used for the identification and quantitative determination of periodate-oxidized ribonucleosides and RNA molecules.[12,28] The resulting derivatives are usually rather stable at neutral and slightly acidic pH values but are hydrolyzed under basic conditions in the presence of ammonia or primary amines.[12] In contrast to hydrazides, the reactions with alkyl hydrazines, semicarbazide, or with thiosemicarbazide sometimes result in the formation of complex mixtures including acyclic mono- and bis-hydrazones and other products depending on the nature of initial reagents and the reaction conditions.[12] The excess of hydrazines can even lead to decomposition of adducts and modified nucleoside residues as well.[10,12,29]

Thus the hydrazone conjugation chemistry in solution provides a simple and practically advantageous route for the generation of numerous ON probes. Essentially any aldehyde-modified reporter label can be tagged to hydrazide ONs.[4] In the case when the modification occurs at the terminus of an ON, it does not interfere at all with base-pairing interactions. For non-terminal modifications, the synthesis of a nucleoside analogue containing a hydrazide derived imidazole residue as a nucleobase was reported.[27] It can be incorporated into ONs both chemically and enzymatically. Conjugation with aldehydes permits the synthesis of a large variety of novel nucleobases in a single step. The coupling reaction of this hydrazide moiety with several aromatic aldehydes was reported to proceed very fast (in several minutes) under the conditions chosen (at pH 4) with high yield.[27]

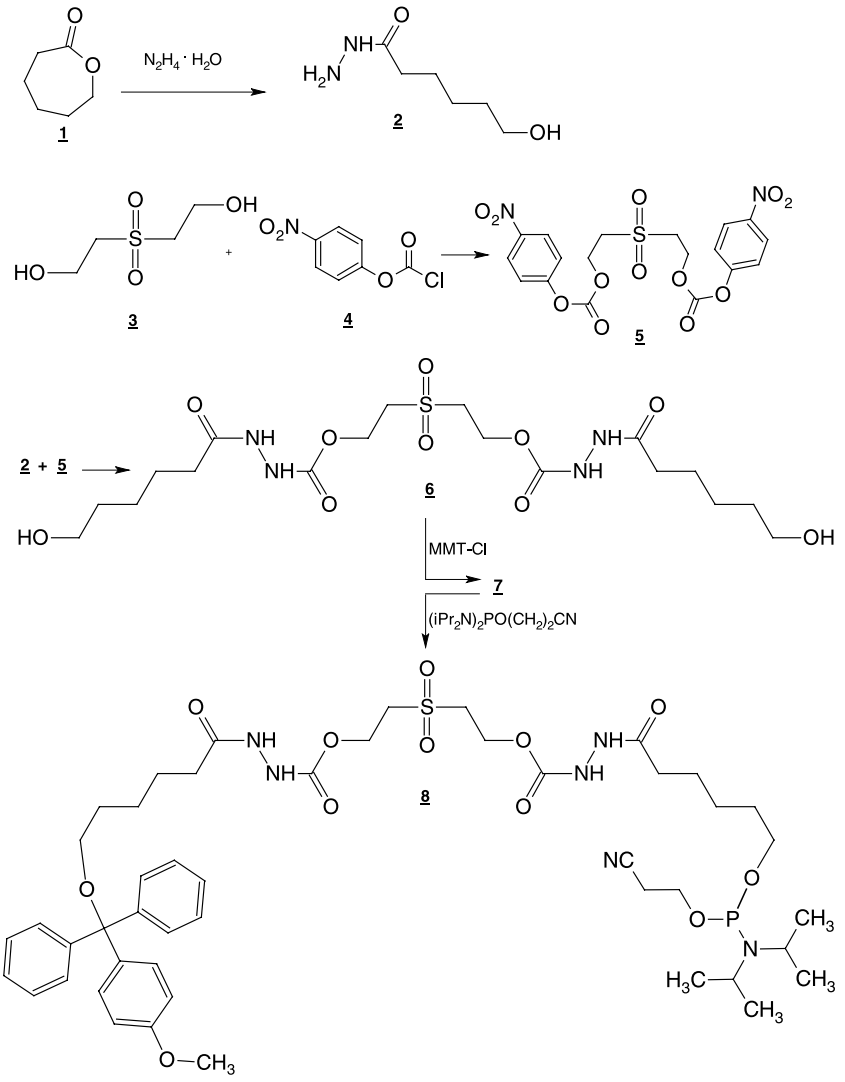
In summary, hydrazide-functionalized ONs are undoubtedly very promising compounds, which clearly will find more and more applications in the near future. At the present time, most known methods propose to the inclusion of the hydrazide moiety into ON post-synthetically.[3,4] Nevertheless, in our opinion automated phosphoramidite-based synthesis can be considered as a most convenient method to introduce hydrazide moieties into ONs. For this purpose appropriately functionalized phosphoramidites and/or solid supports must be used. Recently DMT and trityl protected hydrazide phosphoramidites were described for the 5′-functionalization of ONs.[1,30] Nevertheless according to our knowledge, methods to introduce hydrazide moieties into 3′-end of ONs employing standard amidites in the process of automated chemical synthesis are still unknown. This stimulated us to design a versatile phosphoramidite building block enabling the synthesis of either 3′- or 5′-hydrazide modified ONs or even both in one sequence on the same support.

RESULTS

Our hydrazide modifier **8** (Scheme 2) was synthesized from readily available compounds **1**, **3**, and **4**. The synthetic route is straightforward and proved to be rather efficient and easily scalable.

First, the 6-hydroxyhexanoyl hydrazide (**2**) was obtained by treatment of 6-caprolactone monomer (**1**) by hydrazine hydrate. Bis-2-hydroxyethylsulfone (**3**) was derivatized with a double excess of 4-nitrophenylchloroformate (**4**) and activated derivative **5** was treated by the hydroxyhydrazide **2** to yield the key adduct **6**. The latter was monomethoxytritylated to yield derivative **7** and then phosphitylated with 2-cyanoethyl-N,N,N′,N′-tetraisopropyl phosphane to give the final phosphoramidite derivative **8**.

6-Hydroxyhexanoylhydrazide (**2**) was obtained with quantitative yield. Among a wide variety of derivatizing reagents, 4-nitrophenylchloroformate (**4**) was chosen due to high reactivity and facile work-up conditions. No degradation of active compound **5** was detected during washings or following flash chromatography. Adduct **6** was synthesized in DMF as a solvent due to hydrophilic nature of both reagents and a product; DMAP was added as a catalyst of acylation in the reaction



SCHEME 2 The synthesis of hydrazide modifier 8.

mixture. The MMT group was chosen for hydroxyl protection instead of DMT to provide additional stability to the final derivative. Bannwarth's reagent was employed as a selective and mild phosphitylating reagent, thus avoiding tertiary amines in the reaction mixture, which may provoke the decomposition of derivatives **7** and **8** by β-elimination.[31,32]

Phosphoramidite **8** proved to be a straight-forward modifier in the synthesis of hydrazide modified ONs. A standard protocol of ODN synthesis could be applied except for the introduction of the modifier itself which required slightly longer coupling times (4 min) and increased concentration of phosphoramidite solution

TABLE 1 Sequences of Hydrazide Modified ODNs Obtained

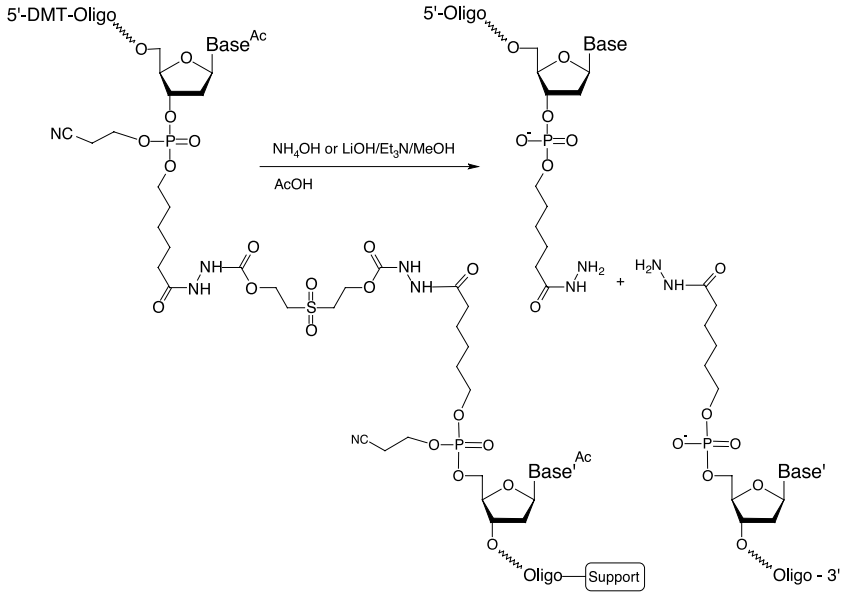
No	Abbr.	Length	ODN sequence (5′ → 3′)
1	AS1	10	TTT TTT TTT T ***X***
2	AS2	13	***X*** ATT AGA CGC CAT C
3	AS3	15	***X*** ATT AGA CGC CAT CTA
4	AS4	15	***X*** TGG TAG CCG CTA GAT
5	AS5	15	***X*** ATT ATG GAC CGA TTT
6	AS6	11	***X*** ATT ATG GAC CG
7	AS7	11	ATT ATG GAC CG ***X***
8	ASK4	15	ATC TAG CGG CTA CCA ***X***
9	ASK5	15	AAA TCG GTC CAT AAT ***X***
10	ASK3	15	TAG ATG GCG TCT AAT ***X***
11	ASK32	15	***X*** TAG ATG GCG TCT AAT ***X***
12	AS32	15	***X*** ATT AGA CGC CAT CTA ***X***
13*	ASD1 (ASK4 & AS6)	15 & 11	ATC TAG CGG CTA CCA ***X*** ATT ATG GAC CG
14*	ASD2 (ASK5 & AS2)	15 & 13	AAA TCG GTC CAT AAT ***X*** ATT AGA CGC CAT C
15*	ASD3 (AS7 & AS4)	11 & 15	ATT ATG GAC CG ***X*** TGG TAG CCG CTA GAT

X is a residue of hydrazide modifier.

*Combination sequences splitting into some of ODNs # 1–12 during post-synthetic work-up.

(0.12 M) to achieve a coupling yield of >90%. We observed a minor decrease of coupling efficiency for the subsequent standard amidites. The latter was, however, not considered as a problem requiring exhaustive optimization.

Fifteen modified ONs with different length and sequence were synthesized employing phosphoramidite **8** (Table 1). The standard post-synthetic procedure



SCHEME 3 The formation of hydrazide containing aligonucleotides upon post-synthetic procedure. Ac: an acyl protecting group; support: Primer Support (Pharmacia); Base, Base': Ade, Gua, Thy, Cyt.

was applied to deprotect modified ODNs and cleave them from solid support (Scheme 3). The structures of oligonucleotides obtained were confirmed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis.

We expected that a side reaction caused by ammonia nucleophilic attack at the carbonyl group of the hydrazide moiety could probably take place. It may yield side products containing an amide or an acid functionality instead of the hydrazide. But actually only a minor amount of side products during deprotection with aqueous ammonia was detected by MALDI TOF MS and HPLC. The formation of side products depends on the time of ammonia treatment and the concentration of ammonia solution as well as on the temperature of the process. As a rule of thumb we used 5 h at 55°C for conc. ammonia and did not observe more than 5–10% of side products. Further shortening of ammonia treatment did not result in a significant improvement. We did not test a more labile protection scheme such as possible using the commercially available PAC phosphoramidites to enable a shortening of ammonia treatment under milder conditions. The latter, however, should allow a decrease in the amount of side products down to trace levels. Also, with such phosphoramidites it should be possible to use hydrazine-based deprotection since the typical side reaction of standard amidites, hydrazination of the N4-position of benzoyl cytidine, can be avoided here due to the nature of the protection groups. It was reported, that the attack of hydrazine either at the amide carbonyl group or at the C4 of the pyrimidine ring depends on the electronic structure of the amide.[33,34]

As a further deprotection method we investigated the procedure originally developed by Mirzabekov et al. for processing of ODNs synthesized on a biochip surface.[35] It includes the treatment of support-bound ODNs by lithium hydroxide

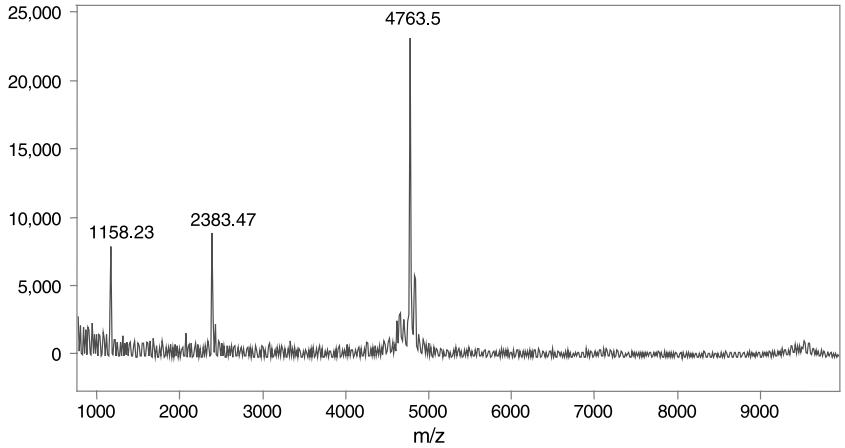


FIGURE 1 MALDI TOF MS analysis of 5′-hydrazide modified ODN # 3 (AS3, 5′-**X**-ATT AGA CGC CAT CTA-3′). **X** is a residue of hydrazide modifier. MW calculated for [M + Na]−: 4765.10; found: 4763.50. For conditions see Experimental.

in the presence of triethylamine and methanol. We considered this procedure to be advantageous in our case as it circumvents ammonia treatment. We found, however, that the outcome is rather similar to ammonia deprotection.

Most of the ODNs were synthesized in trityl-ON mode. ODNs containing modification on the 3′-end were purified by reverse-phase HPLC using the hydrophobic properties of DMT group. ODNs containing the hydrazide modification at the 5′-end do not carry any hydrophobic protecting group. Nevertheless, it was found that it is possible to purify these ODNs by reversed phase HPLC due to presence of a residue of the hydrazide modifier on 5′-terminus which causes substantial shift of retention time. All ONs were synthesized in 1.3 μmole scale, but the whole procedure is easily scalable. Under the conditions chosen the overall yield of hydrazide ODNs was typically not less than 50%. Retention times of modified ODNs proved to be different from those of unmodified ODNs of the same sequences,

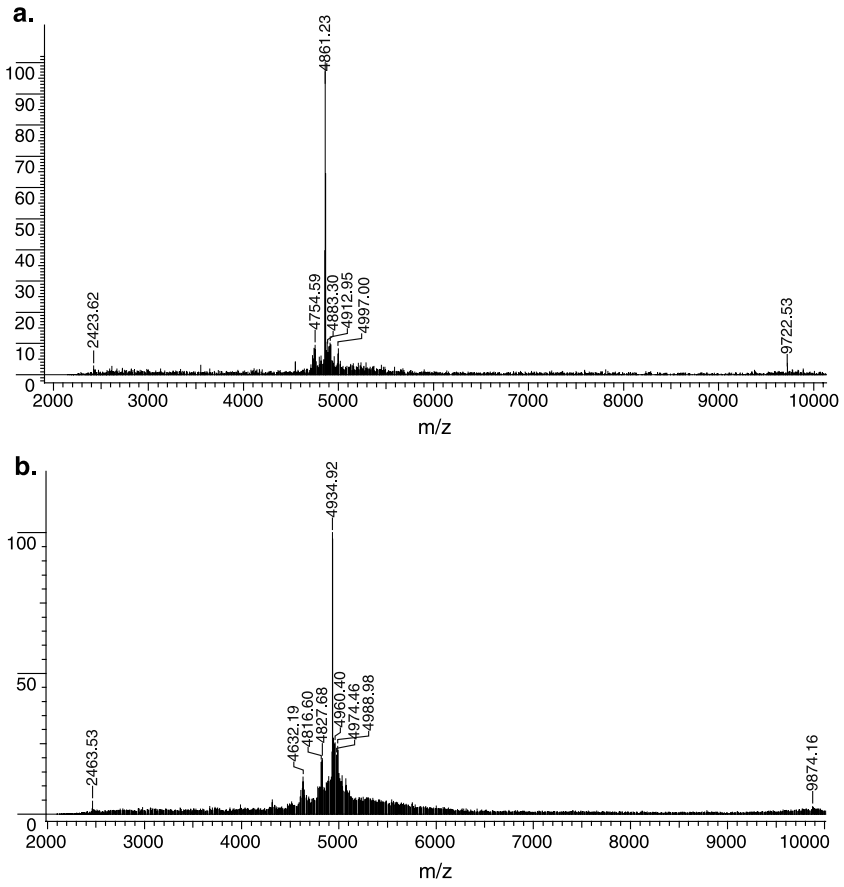


FIGURE 2 MALDI TOF MS analyses of conjugates of 5′-modified ODNs with anisaldehyde. **a.** Conjugate of ODN # 3 (AS3, 5′-**X**-ATT AGA CGC CAT CTA-3′) with anisaldehyde. MW calculated for $[M]^-$: 4861.25; found: 4861.23. **b.** Conjugate of ODN #4 (AS4, 5′-**X**-TGG TAG CCG CTA GAT-3′) with anisaldehyde. MW calculated for $[M]^-$: 4933.30; found: 4934.92. **X** is a residue of hydrazide modifier. For conditions see Experimental.

both for DMT-containing ODNs and for ODNs without the DMT group. The identities of synthetic ODNs containing the hydrazide functionality were confirmed by MALDI TOF MS (Figure 1). Analysis using negative-ion MALDI TOF MS revealed experimental masses that are in agreement with theory.

The additional reliable evidence of the presence and chemical reactivity of the hydrazide moiety in ODNs came from the successful conjugation with anisaldehyde. The reaction was found to proceed rather fast and could be monitored by reverse-phase HPLC. The conjugates of modified ODNs with anisaldehyde were isolated by reverse-phase HPLC and structures were confirmed by MALDI TOF MS analysis (Figure 2).

EXPERIMENTAL PART

General

Unless otherwise indicated, all reactions were performed under dry argon. Reagents were obtained from Aldrich or Acros Organics Chemical Companies. Column chromatography was accomplished using silica gel 60, 230–400 mesh (Merck). ODN samples were evaporated using a Speed Vac Plus 110 A concentrator (Savant).

^{1}H-NMR spectra were recorded at 200.131 MHz with the Bruker DPX-200 spectrometer. ^{1}H-NMR spectra are reported in units of δ using tetramethyl silane as an internal standard and coupling constants are reported in units of Hz. ^{1}H-NMR spectrum of the phosphoramidite **8** was recorded at 400.131 MHz and its ^{31}P-NMR spectrum was recorded at 161.992 MHz with the Bruker DRX-400 spectrometer. ESI mass spectra were recorded with the Bruker Esquire LC 00153 spectrometer in positive ionization mode. The MALDI TOF MS measurements were performed with the Perseptive Voyager DE-RP and the Bruker Daltonics Autoflex instruments in negative ionization mode. Samples were irradiated with a nitrogen laser operated at 337 nm. Ions produced by laser desorption were energetically stabilized during a delayed extraction period of 300 nanoseconds and then accelerated through the linear TOF mass analyzer by 25 kV potential pulse or by 20kV in Bruker Autoflex. As a matrix 3-hydroxypicolinic acid in a mixture of acetonitrile and water (1:1 v/v) was used.

HPLC analyses and purifications were performed with Kontron HPLC system in reverse-phase mode. The CC 250/4 Nucleodur 100-5 C18 ec column (Macherey-Nagel) was used (size 4 × 250 mm, particle size 7 μm). Flow rate 1.0 ml/min at room temperature was applied. 0.1 M Ammonium hydrogen carbonate buffer was used as mobile phase A and 100% acetonitrile was used as mobile phase B. For DMT group containing ODNs usually the gradient of 5% to 35% of mobile phase B in 30 min (1% of mobile phase B per minute) was used; for ODNs without DMT group usually the gradient of 5% to 20% of mobile phase B in 30 min (0.5% of mobile phase B per minute) was used.

The Synthesis of Phosphoramidite Building Block for the Incorporation of Hydrazide Into ONs

6-Hydroxyhexanoylhydrazide (2). Two hundred millimoles of aqueous hydrazine (35 wt. %) was slowly added to a solution of 21.2 ml (200 mmole) of 6-caprolactone monomer (**1**) in 200 ml of ethanol under vigorous stirring. The reaction mixture was heated to reflux for 12 h and then cooled to room temperature. The obtained colorless crystals were recrystallized from hot ethanol and dried under vacuum to afford 28.4 g (97%) of 6-hydroxyhexanoylhydrazide (**2**). ^{1}H-NMR (D_2O): 1.31 (2H, m, CH_2–$(CH_2)_2$–OH); 1.57 (4H, m, CH_2–CH_2–CH_2–CH_2–OH); 2.21 (2H, t, CO–CH_2, J=7.26 Hz); 3.58 (2H, t, CH_2–OH, J=6.44 Hz). ESI MS: $C_6H_{14}N_2O_2$, $[M + Na]^+$ calculated: 169.18; found: 169.1.

Bis-[(4-nitrophenyl)oxycarbonyl-2-oxyethyl]sulfone (5). Twenty-five grams (124 mmol) of 4-nitrophenylchloroformate (**4**) was added to a solution of 8.63 g (56 mmol) of bis-2-hydroxyethylsulfone (**3**) in 300 ml of anhydrous methylene chloride. Then 10.05 ml (125 mmol) of anhydrous pyridine was slowly added dropwise under ice cooling and vigorous stirring. The reaction mixture was vortexed for 1 h at room temperature. The precipitate was filtered, washed twice with water, and dried under vacuum. A second fraction was obtained from the organic phase. It was washed twice with water and dried over anhydrous sodium sulfate. Organic phase and precipitate were combined and concentrated in vacuo. The residue was purified by flash column chromatography and eluted with a step gradient of 0–25% (v/v) of ethanol in methylene chloride. Product containing fractions were concentrated and dried in vacuo yielded 17.67 g (66.4%) of compound **5**. ^{1}H-NMR (DMSO-D_6): 3.75 (4H, t, CH_2–SO_2); 4.66 (4H, t, OCO–CH_2, J=5.56 Hz); 7.55 (4H, d, OCO–C–*CH*); 8.31 (4H, d, NO_2–C–*CH*, J=8.71 Hz). ESI MS: $C_{18}H_{16}N_2O_{12}S$, $[M + NH_4]^+$ calculated: 502.43; found: 503.1.

Bis-[(6-hydroxyhexanoyl)hydrazinocarbonyl-2-oxyethyl]sulfone (6). To a solution of 5.95 g (40.7 mmol) of 6-hydroxyhexanoylhydrazide (**2**) in 350 ml of anhydrous DMF, 9.7 g (20 mmol) of bis-[(4-nitrophenyl)oxycarbonyl-2-oxyethyl]sulfone (**5**) was added. Then, 1620 µl (20 mmol) of anhydrous pyridine was slowly added dropwise under vigorous stirring followed by the addition of 0.05 eq. of DMAP. The reaction mixture was vortexed during 20 h at room temperature. The DMF was evaporated in vacuo and 200 ml of methylene chloride was added to reaction mixture, followed by 10 ml of acetic acid and 200 ml of water. The product was extracted with water. The aqueous layer was washed once with 50 ml of methylene chloride with 3 ml of acetic acid. The solution was evaporated in vacuo and coevaporated twice with anhydrous acetonitrile. The product was recrystallized from hot acetonitrile and dried in vacuo to afford 6.5 g (65.2%) of compound **6**. ^{1}H-NMR (D_2O): 1.38 (4H, m,

CH$_2$–(CH$_2$)$_2$–OH); 1.58 (8H, m, *CH*$_2$–CH$_2$–*CH*$_2$–CH$_2$–OH); 2.31 (4H, t, CO–*CH*$_2$, J=7.07 Hz); 3.59 (4H, t, *CH*$_2$–SO$_2$, J=6.37 Hz); 3.68 (4H, bs, *CH*$_2$–OH); 4.61 (4H, t, OCO–*CH*$_2$). ESI MS: $C_{18}H_{34}N_4O_{10}S$, $[M + Na]^+$ calculated: 521.54; found: 521.2.

6-Monomethoxytrityloxy-6′-hydroxy-bis-(hexanoylhydrazinocarbonyl-2-oxyethyl)sulfone (7). The solution of 2.93 g (9.5 mmol) monomethoxytrityl chloride in 100 ml of anhydrous methylene chloride was slowly added dropwise, while stirring vigorously, to a solution of 5 g (10 mmol) of bis-[(6-hydroxyhexanoyl)hydrazinocarbonyl-2-oxyethyl]sulfone (**6**) in 120 ml of anhydrous pyridine. The reaction mixture was vortexed during 3 h at room temperature. The solvents were evaporated in vacuo and 100 ml of ethyl acetate was added. The organic phase was washed once with 100 ml of brine, once with 100 ml of water, and dried over anhydrous sodium sulfate. After evaporation of the solvent in vacuo the residue was purified by flash column chromatography, eluting with a step gradient of 0–5% (v/v) of ethanol in methylene chloride containing 0.5% (v/v) of pyridine. Product containing fractions were concentrated, coevaporated with 20 ml of benzene for three times and dried in vacuo yielding 3.64 g (49.7%) of compound **7**. ^{1}H-NMR (DMSO-D$_6$): 1.33 (4H, m, *CH*$_2$–(CH$_2$)$_2$–OH, *CH*$_2$–(CH$_2$)$_2$–O–MMT); 1.50 (8H, m, *CH*$_2$–CH$_2$–*CH*$_2$–CH$_2$–OH, *CH*$_2$–CH$_2$–*CH*$_2$–CH$_2$–O–MMT); 2.07 (4H, bs, NHCO–*CH*$_2$); 2.92 (4H, t, *CH*$_2$–SO$_2$, J=6.00 Hz); 3.54 (4H, bt, *CH*$_2$–OH, *CH*$_2$–O–MMT); 3.73 (3H, s, O–*CH*$_3$); 4.34 (4H, t, OCO–*CH*$_2$); 6.88 (2H, d, CH$_3$O–C–*CH*); 7.23 (2H, d, CH$_3$O–C–CH=*CH*, J=8.53 Hz); 7.34 (10H, m, *C*$_6$*H*$_5$). ESI MS: $C_{38}H_{50}N_4O_{11}S$, $[M + NH_4]^+$ calculated: 788.93; found: 788.4.

6-Monomethoxytrityloxy-6′-[(2-cyanoethoxy)(diisopropylamino)phosphanyloxy]-bis-(hexanoylhydrazinocarbonyl-2-oxyethyl)sulfone (8). To a solution of 0.463 g (0.6 mmol) of 6-monomethoxytrityloxy-6′-hydroxy-bis-(hexanoylhydrazinocarbonyl-2-oxyethyl)sulfone (**7**) and 0.334 g (1.95 mmol) of diisopropylamidotetrazolide in 15 ml of anhydrous methylene chloride, a solution of 572 µl (1.8 mmole) of 2-cyanoethyl-N,N,N′,N′-tetraisopropyl phosphane in 10 ml of anhydrous methylene chloride was added dropwise via a syringe over a period of 5 min under vigorous stirring. The reaction mixture was vortexed for 2 h at room temperature. Then mixture was diluted with 125 ml of ethyl acetate and washed once with 150 ml of brine and once with 150 ml of water. The organic phase was dried over anhydrous sodium sulfate, evaporated in vacuo, and dried under vacuum, affording 0.54 g (92.7%) of compound **8** as a light-yellow foam. As TLC analysis showed complete consumption of initial compound **7**, the resulting phosphoramidite **8** was used in ODN synthesis without further purification. ^{1}H-NMR ($CDCl_3$): 1.18 (12H, bd, ((*CH*$_3$)$_2$CH)$_2$N-, J=6.52 Hz); 1.24; 1.29; 1.40; 1.62 (12H, m, *CH*$_2$–*CH*$_2$–*CH*$_2$–CH$_2$–O–P, *CH*$_2$–*CH*$_2$–*CH*$_2$–CH$_2$–O–MMT); 2.23 (4H, bt, NHCO–*CH*$_2$, J=5.52 Hz);

2.60 (2H, t, NC–CH_2–CH_2–O–, J=6.02 Hz); 3.06 (4H, bt, CH_2–SO_2, J=6.53 Hz); 3.30; 3.42; 3.58 (6H, m, $((CH_3)_2CH)_2$N–P, $(CH_2)_4$–CH_2–O–P, $(CH_2)_4$–CH_2–O–MMT); 3.71 (2H, m, NC–CH_2–CH_2–O); 3.80 (3H, s, O–CH_3); 4.54 (4H, bt, OCO–CH_2); 7.21; 7.27; 7.42 (14H, m, C_6H_4, C_6H_5). ^{31}P-NMR ($CDCl_3$): 147.49. ESI MS: $C_{47}H_{67}N_6O_{12}PS$, $[M + H]^+$ calculated: 972.12; found: 971.3.

The Synthesis of Hydrazide Containing ONs

ODNs were synthesized using solid-phase phosphoramidite chemistry on an automated nucleic acids synthesizer Gene Assembler from Pharmacia Biotech in a 1.3 μmol scale. "Primer Supports" from Pharmacia Biotech (50–70 μm solid-phase polystyrene bead matrix) were used as solid support. 4,5-Dicyanoimidazole in acetonitrile was used as activator. The hydrazide containing phosphoramidite **8** was applied as a 0.12 M solution in anhydrous acetonitrile. It was coupled at desired positions in ODN sequence using 4 min coupling time. No other changes were made in the standard protocol of phosphoramidite ODN synthesis.

Deprotection and Isolation of Hydrazide Containing ODNs

Two different deprotection and work-up strategies were applied.

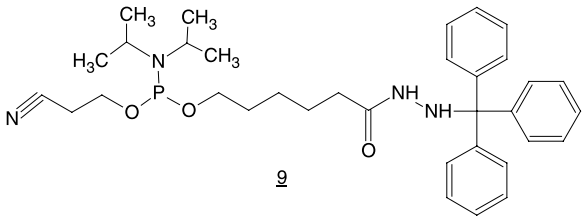
Standard Protocol

The solid support bound ODN was placed in a 2-ml microtube with screw cap (Sarstedt) and treated by 0.5 ml of 25% aqueous ammonia solution at 55°C. After 5 h the test tube was cooled to room temperature, ammonia solution was removed, evaporated to dryness under reduced pressure, and the residue was redissolved into 0.5 ml of water.

Alternative Protocol

The solid support bound ODN was placed in a 2-ml test tube and 30 μl of 0.5 M solution of lithium hydroxide in water and then 300 μl of 3.5 M solution of triethylamine in methanol were added. The reaction mixture was vortexed during 1 h at 75°C. 75 μl of glacial acetic acid was added at room temperature and the solution was cooled at − 20°C for 5 min. After centrifugation the supernatant was discarded, the precipitate was washed twice with 90% (v/v) acetonitrile in water, twice with acetonitrile, and then redissolved in 0.5 ml of water.

The DMT group containing ODNs were analyzed and purified by reverse-phase HPLC using the conditions described above. The DMT protecting group was removed by treating the ODN with 80% acetic acid for 30 min at room temperature. 500 μl of 50% (v/v) aqueous methanol was added to the acidic solution which was then evaporated in vacuo. ODN was redissolved in 0.5 ml of water. ODNs with free 5′-hydroxyl group were analyzed and purified by reversed phase HPLC using the conditions described above.



9

SCHEME 4 Phosphoramidite modifier developed by Nanogen Recognomics.

Conjugation of Hydrazide Containing ODNs and Aldehyde

Five nanomoles of the hydrazide containing ODN was evaporated to dryness and then dissolved in 10 μl of 0.1 M MES buffer (pH 5.0–5.3), 4 μl of a 1 M solution of 4-methoxyphenylaldehyde in DMF was added. After 30 or 60 min at 30 to 37°C, ODN was desalted in NAP-10 Sephadex cartridge (Pharmacia Biotech). An aqueous solution of ammonium acetate (1 mM) was used for the elution. The ODN solution was concentrated under reduced pressure to a volume of 0.4 ml and the reaction mixture was analyzed by reverse-phase HPLC in conditions described above. The conjugates were isolated from the reaction mixtures under the same conditions. Fractions containing conjugates were collected, evaporated to dryness in vacuo, redissolved in 500 μl of water, and analyzed by HPLC and MALDI TOF MS using conditions as described above.

CONCLUSIONS

Our method for the introduction of the hydrazide moiety into ONs was found to be rather efficient. It is based on a versatile phosphoramidite building block **8** containing two hydrazide residues connected to β-eliminable unit, whose synthesis (Scheme 2) is quick and straight-forward, starting from inexpensive commercially available building blocks. Our phosphoramidite modifier can be employed in the standard ON synthesis process and yield hydrazide containing ONs upon ammonia or lithium hydroxide treatment. The hydrazide functions are liberated under the mild conditions of a β-elimination yielding only low amount of side products. In most cases the hydrazide modified ONs should nevertheless be purified using reverse-phase HPLC. For the case of 3′-modified ONs it is further recommended to keep the 5′-DMT group "on," especially if one uses our method to prepare in a single run two hydrazide-modified ONs of similar length. For the case of 5′-modified ODNs, which are obtained without a lipophilic DMT group, it was nevertheless quite easy to achieve the required level of ON purity using reverse-phase HPLC.

Recently, 5′-hydrazide modified ONs became commercially available from *Metabion* company. Their synthesis is based on the modifier amidite **9** (Scheme 4) developed by *Nanogen Recognomics GmbH*,[1] which formally allows to introduce

the hydrazide moiety also at the 3′-end when employing reversed phosphoramidites whose cost and coupling efficiency limit a wider applicability.

Our modifier has the advantage of being compatible with standard ON-synthesis when 3′-modified ONs are required. Both 3′-and 5′-modified ONs are easily accessible using our hydrazide modifier and may find particular applications in the field of DNA nanotechnology.[36–38]

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