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N-Methoxy-1,3-Oxazinane Nucleic Acids (MOANAs) – a Configurationally Flexible Backbone Modification Allows Post-Synthetic Incorporation of Base Moieties

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Abstract

(2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol was converted into a protected phosphoramidite building block and incorporated into the middle of a short DNA oligonucleotide. O1 and O3 of the (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol were engaged in phosphodiester linkages, leaving O2 and the methoxyamino function available to form an *N*-methoxy-1,3-oxazinane ring through reaction with an aldehyde. In modified oligonucleotides thus obtained, the oxazinane ring formally replaces the furanose ring and the aldehyde the base moiety of natural nucleosides. The feasibility of synthesizing base-modified oligonucleotides by this approach was demonstrated with several aromatic and aliphatic aldehydes featuring various functional groups.

Introduction

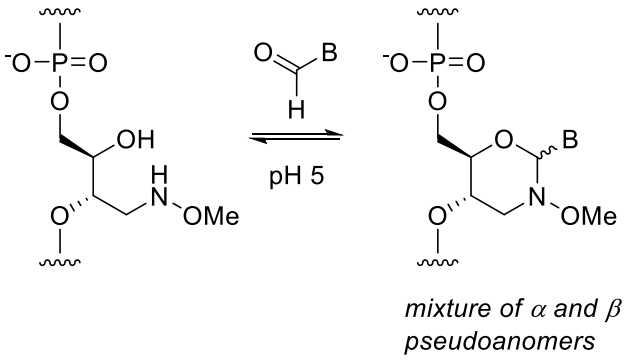
Modified or completely artificial base moieties can imbue oligonucleotides with new properties, such as alternative base pairing patterns[1–16], catalytic activity[17,18] or the ability to bind metal ions, either coordinatively[19–24] or through formation of a carbon—metal bond[25,26]. Unnatural base moieties are most commonly introduced into oligonucleotides as phosphoramidite building blocks of the corresponding nucleoside analogues by automated solid-phase synthesis. This method, while undeniably robust and elegant, is rather labor-intensive and regio- and stereospecific glycosidation of a given base moiety can hardly be considered a routine task. Furthermore, some structures are obviously incompatible with the conditions of the phosphoramidite strategy. The other popular approach, namely enzymatic polymerization, also suffers from the laborious preparation of

nucleoside analogues, in addition to being somewhat limiting in terms of the modifications tolerated by the polymerase. For screening of large numbers of unnatural nucleobases, a method for their post-synthetic introduction into a common oligonucleotide scaffold would be highly desirable.

A plethora of coupling chemistries have been employed successfully for post-synthetic furnishing of oligonucleotides with various conjugate groups.[27–29] Most of these approaches are sufficiently robust and modular to allow generation of reasonably large screening libraries. However, the linkages formed tend to be rather bulky and in many cases stereochemically nonhomogeneous. In many applications, such as labeling or affinity tagging, this is not a major problem. Post-synthetic introduction of base moieties, however, places stricter demands on the conjugation chemistry as the linkage between the oligonucleotide backbone and the base moiety should be small enough to allow undisrupted stacking of the latter within the double-helical environment. Ideally, a close structural mimic of the natural nucleosides should by formed. Of the commonly used conjugation reactions, oximation yields the smallest linkage and we have had some success using it for the incorporation of metallacyclic benzaldehydes into aminooxy-functionalized oligonucleotides.[30,31] However, even in that case the sugar and base moieties were separated by an additional oxymethylene linker. With a PNA backbone, “base filling” through peptide coupling and reductive amination has met with considerable success[32] but, to the best of our knowledge, the feasibility of a similar strategy with a sugar phosphate backbone remains to be demonstrated.

In this study, we present a new nucleic acid backbone modification designed for post-synthetic incorporation of base moieties. In this modification, (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol units, linked through O1 and O3 by conventional phosphodiester bonds, form the backbone and the base moieties are introduced as aldehydes through formation of an *N*-methoxy-1,3-oxazinane with O2 and N4 (Scheme 1). The aldehydes could be either formyl-functionalized analogues of the canonical nucleobases or completely artificial structures. According to previous reports on *N*-methoxyoxazolidines[33], the reaction is reversible at pH 5 but essentially irreversible at pH 7, allowing the base moieties to bind under conditions where the rules of Watson—Crick base pairing apply. The resulting *N*-methoxy-1,3-oxazinane nucleic acids (MOANAs) are isosteric with the previously reported hexopyranose nucleic acids[34–37] featuring 1-(2,3-dideoxy-D-*erythro*-hexopyranose) as the sugar unit. The β anomer of this so-called *homo*-DNA does not form a stable duplex with natural nucleic acids[35] but the α anomer readily hybridizes with RNA, albeit in a parallel fashion[38]. In this regard it is interesting to point out that the pH-controlled configurational flexibility of the MOANA backbone also allows equilibration of the α and β pseudoanomers under conditions

where the Watson—Crick base pairing rules apply. The MOANA backbone can, in a sense, be considered as an intermediate between cyclic and acyclic[39–41] nucleic acid backbones.



Scheme 1. Incorporation of a base moiety through formation of an *N*-methoxy-1,3-oxazinane ring.

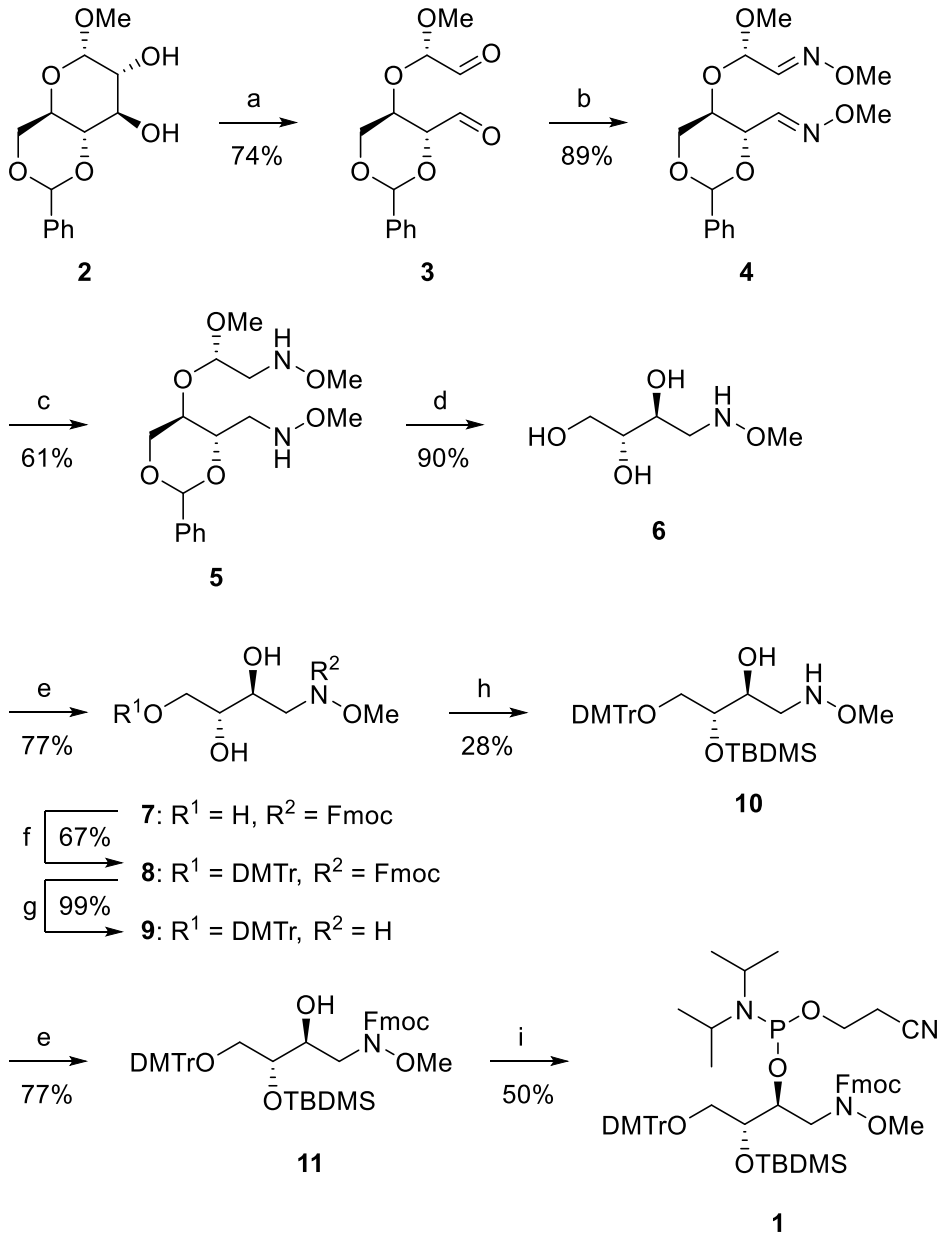
Results and discussion

Building block synthesis

Synthesis of the protected (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol phosphoramidite building block **1** is presented in Scheme 2. The synthesis started from commercially available methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**2**), which was oxidized following a previously reported protocol[42] to yield the dialdehyde **3**. The aldehyde functions were then oximated by treatment with methoxyamine hydrochloride in pyridine to yield compound **4** as a mixture of geometric isomers. Reduction of this mixture with sodium cyanoborohydride in a mixture of acetic acid, dichloromethane and methanol afforded compound **5**. The acetal protections were cleaved in 6 M hydrochloric acid in the presence of 1,3-propanedithiol as a scavenger for the released aldehydes. The dithioacetal byproducts were washed away with dichloromethane and the remaining aqueous solution neutralized with strong anion exchange resin to afford (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol (**6**).

The methoxyamino function of compound **6** was first protected as a 9-fluorenylmethyl carbamate by treatment with 9-fluorenylmethyl chloroformate and potassium carbonate in a mixture of water and 1,4-dioxane, yielding compound **7**. The primary hydroxy group of this intermediate was then dimethoxytritylated by treatment with 4,4´-dimethoxytrityl chloride in a mixture of dichoromethane and pyridine to afford compound **8**. Diethylamine-promoted removal of the Fmoc protection in dichloromethane gave the monoprotected intermediate **9** and subsequent

reaction with *tert*-butyldimethylsilyl chloride in *N*,*N*-dimethylformamide in the presence of imidazole a chromatographically separable mixture of the 2- and 3-*O*-silylated products, from which the desired isomer **10** was recovered. The Fmoc protection was then reintroduced on the methoxyamino group under the abovementioned conditions, yielding compound **11**. The tedious Fmoc removal and reintroduction was unavoidable as this protection was found to be somewhat labile under the silylation conditions. Furthermore, compound **11** and its 3-*O*-silylated isomer proved chromatographically inseparable. Finally, the remaining hydroxy function of compound **11** was phosphitylated by conventional treatment with 2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite and triethylamine in dichloromethane under nitrogen atmosphere to afford the desired phosphoramidite building block **1**.



Scheme 2. Synthesis of the protected (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol phosphoramidite building block **1**. Reagents and conditions: a) $NaIO_4$, MeOH, H_2O, 25 °C, 20 h; b) $MeONH_3Cl$, pyridine, 80 °C, 16 h; c) $NaCNBH_3$, AcOH, CH_2Cl_2, MeOH, 25 °C, 16 h; d) HCl, 1,3-propanedithiol, H_2O, 40 °C, 16 h; e) FmocCl, K_2CO_3, 1,4-dioxane, H_2O, 25 °C, 16 h; f) DMTrCl, CH_2Cl_2, pyridine, N_2 atmosphere, 25 °C, 16 h; g) Et_2NH, CH_2Cl_2, 25 °C, 45 min; h) TBDMSCl, imidazole, DMF, 25 °C, 16 h; i) 2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite, Et_3N, CH_2Cl_2, N_2 atmosphere, 25 °C, 45 min.

Oligonucleotide synthesis

Phosphoramidite building block **1** was incorporated into the middle of an 11-mer oligodeoxynucleotide **ON1** (5´-CGAGCXCTGGC-3´, where X refers to the modified residue) by phosphoramidite strategy on an automated synthesizer. The coupling yield of **1** was only approximately 64% despite a prolonged coupling time of 15 min, while the other couplings proceeded with normal (>99%) efficiency. Release from solid support and removal of the base and phosphate protections, as well as the Fmoc protection of the methoxyamino group of **1**, was achieved by overnight incubation in 25% aqueous ammonia at room temperature. The crude product thus obtained was first purified by RP-HPLC with the TBDMS protection on to facilitate separation of the full-length and truncated sequences. After desilylation by the conventional treatment with triethylamine trihydrofluoride in dimethylsulfoxide, the oligonucleotide was passed through an RP-HPLC column again to yield the pure product. The identity of **ON1** was verified mass spectrometrically and the concentration determined spectrophotometrically. In addition to naked **ON1**, 12 and 26 Da higher masses were observed, in all likelihood resulting from the reaction of **ON1** with formaldehyde and acetaldehyde impurities of the solvents used. As this side product was expected to be in dynamic equilibrium with **ON1**, it was not expected to present a problem when studying the reactivity of **ON1** with other aldehydes. Apart from reactions with aldehyde impurities, **ON1** was found to be stable under conditions relevant for reaction with aldehydes and isolation of the products (*T* = 25 and 55 °, pH = 5.0 and 7.4, experimental details provided in the supporting information). Owing to the hydroxy group vicinal to a phosphodiester linkage, the possibility of transesterification and cleavage reactions similar to those of RNA cannot be ruled out under highly alkaline or acidic conditions.

Derivatization of the oligonucleotide scaffold with aldehydes

The potential of *N*-methoxy-1,3-oxazinane formation for the introduction of artificial base moieties was demonstrated by derivatizing the oligonucleotide scaffold **ON1** with a number of aldehydes, selected to represent both aliphatic and (hetero)aromatic structures, as well as various functional groups. **ON1** and a large excess of the aldehyde were incubated overnight at 55 °C in a mixture of acetonitrile and aqueous acetate buffer (pH = 5.0). The reaction was quenched by adjusting the pH to 7.0, after which the aqueous solution was washed with dichloromethane and fractioned on RP-HPLC. All chromatograms are included in the supporting information and a representative example is

shown in Figure 1. As expected, **ON1** was converted to a slower-eluting product with all aldehydes studied.

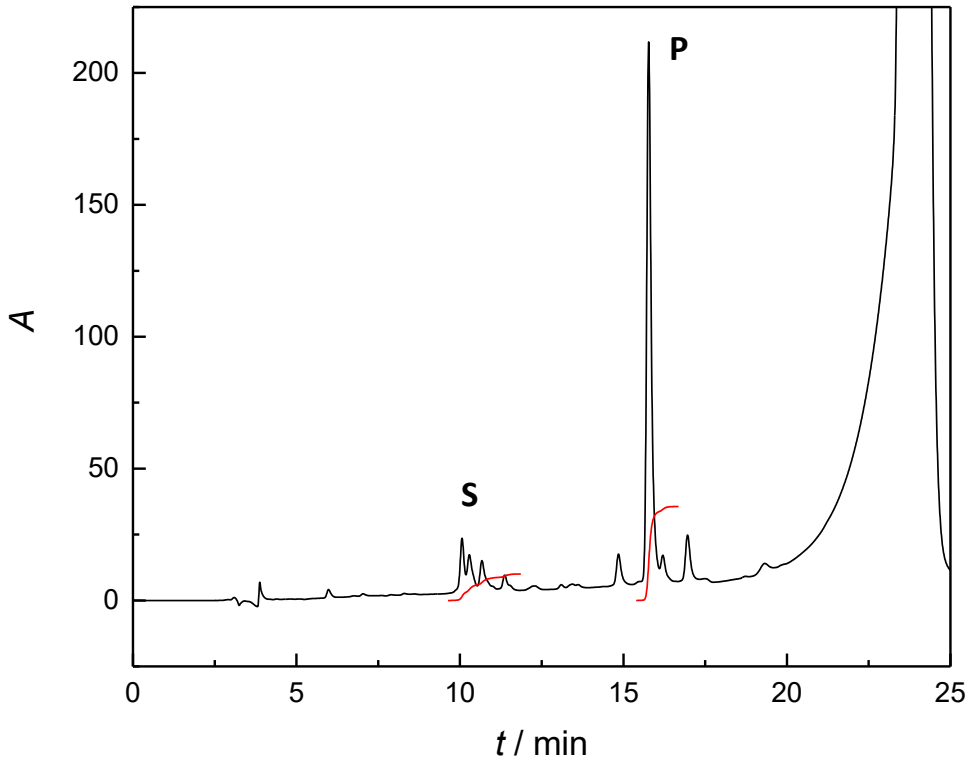


Figure 1. RP-HPLC trace of the product mixture of the reaction of the oligonucleotide scaffold **ON1** with 3-hydroxybenzaldehyde; Hypersil ODS C18 column (250 × 4.6 mm, 5 µm); detection wavelength = 260 nm; flow rate = 1.0 mL min^{-1}; linear gradient (8—23% over 25 min) of acetonitrile in 50 mM aqueous triethylammonium acetate (pH = 7.0). The starting material peaks are indicated by "S" and the product peaks by "P". The large, slow-eluting peak corresponds to unreacted 3-hydroxybenzaldehyde.

Mass spectrometric analysis of the product fractions confirmed formation of the expected derivatives. The yields ranged from 24% to 82%, the lowest ones being obtained with relatively volatile aldehydes, such as benzaldehyde and butyraldehyde. Retention times, yields and calculated and observed *m*/*z* values of the products are summarized in Table 1. Attempts to estimate the ratio of α and β pseudoanomers of the oligonucleotide products based on corresponding reactions of monomeric (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol (**6**) were not successful but ^{1}H NMR analysis of the reaction mixture was consistent with the pseudoanomers existing in rapid equilibrium (experimental details and data presented in the supporting information).

Table 1. RP-HPLC retention times, yields and calculated and observed *m*/*z* values of the products of the reactions of the oligonucleotide scaffold **ON1** with various aldehydes.

Aldehyde	t_R / min[a]	yield / %[b]	calculated *m*/*z*[c]	observed *m*/*z*[c]
benzaldehyde	17.7	24	1663.8	1663.8
3-hydroxybenzaldehyde	15.8	78	1671.8	1671.8
3-methoxybenzaldehyde	17.4	55	1678.8	1678.8
3-cyanobenzaldehyde	17.1	63	1676.3	1676.3
3-nitrobenzaldehyde	17.8	82	1686.3	1686.3
isophthalaldehyde	16.0	71	1677.8	1677.8
imidazole-2-carbaldehyde	11.3	80	1658.8	1658.8
quinoline-2-carbaldehyde	12.7	53	1689.3	1689.3
butyraldehyde	15.9	33	1646.8	1646.8
benzyloxyacetaldehyde	16.6	82	1685.8	1686.3

[a] For HPLC conditions, see Figure 1.

[b] Estimated based on relative peak areas in HPLC.

[c] Bruker Daltonics micrOTOF-Q, $[M - 2H]^{2-}$

HPLC traces of many of the product mixtures showed a large peak of residual aldehyde despite washing with dichloromethane. In some cases, the aldehyde peak overlapped with the starting material or the product oligonucleotide, complicating the purification and making reliable estimation of the yield impossible. In an attempt to improve on this point, we repeated the reactions with 3-hydroxybenzaldehyde, 3-nitrobenzaldehyde and butyraldehyde with a greatly reduced amount of the aldehyde (100 equivalents) but under otherwise the same conditions. With the aromatic aldehydes, a dramatic loss of yield was observed whereas with butyraldehyde the yield actually improved modestly and the HPLC trace of the product mixture was cleaner (all chromatograms are included in the supporting information). Remarkable yields for the related *N*-methoxyoxazolidine formation have been obtained with as little as 1.5 equivalents of the aldehyde by performing the reaction in a mixture of dimethylsulfoxide and acetic acid instead of an aqueous buffer.[33] These conditions were also tested in the present case (with 2.0 equivalents of 3-hydroxybenzaldehyde, 3-nitrobenzaldehyde or butyraldehyde) but with no detectable conversion of the starting oligonucleotide **ON1** (data not shown). Apparently a large excess of the aldehyde is necessary to drive the *N*-methoxy-1,3-oxazinane formation to completion, especially with aromatic aldehydes. On

the other hand, the amounts used are still very small compared to what would be required to introduce modified base moieties by the conventional method (*i.e.* as corresponding nucleoside phosphoramidites).

Reversibility of the *N*-methoxy-1,3-oxazinane formation was studied by diluting purified samples of 3-hydroxybenzaldehyde and 3-cyanobenzaldehyde derivatives of **ON1** with aqueous acetate buffer (pH = 5.0). The diluted samples were incubated overnight at 55 °C and analyzed by RP-HPLC. Disappearance of the aldehyde derivative and appearance of **ON1** (as the acetaldehyde adduct) could be detected in both cases (experimental details and chromatograms presented in the supporting information).

Conclusions

We have developed a synthesis strategy for a protected phosphoramidite building block derived from (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol and incorporated this building block in the middle of a short DNA oligonucleotide. Various derivatives of this oligonucleotide scaffold were synthesized by simple incubation with an aldehyde under aqueous conditions. Reaction of an aromatic aldehyde with (2*R*,3*S*)-4-(methoxyamino)butane-1,2,3-triol affords a configurationally flexible structural mimic of a nucleoside, with (5S,6R)-6-(hydroxymethyl)-*N*-methoxy-1,3-oxazinan-5-ol playing the role of the sugar and the aromatic ring the role of the nucleobase. The resulting *N*-methoxy-1,3-oxazinane nucleic acid (MOANA) backbone could find use in diverse applications, such as modified aptamers, DNAzymes and DNA nanostructures.

Author Contributions

Mark Afari: Investigation, Data Curation, Writing – Original Draft. **Pasi Virta**: Conceptualization, Resources, Writing – Review and Editing, Project Administration. **Tuomas Lönnberg**: Conceptualization, Methodology, Validation, Data Curation, Writing – Review and Editing, Visualization, Supervision, Project Administration.

Conflicts of interest

There are no conflicts to declare.

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