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TITLE	Conjugation of Oligonucleotides to Peptide Aldehydes via a pH-Responsive *N*-Methoxyoxazolidine Linker
YEAR	2020. August 17th.
DOI	https://doi.org/10.1021/acs.orglett.0c01815
VERSION	Final Draft.
CITATION	*Org. Lett.* 2020, 22, 17, 6714–6718 Publication Date:August 17, 2020 https://doi.org/10.1021/acs.orglett.0c01815

Conjugation of oligonucleotides to peptide aldehydes via pH-responsive N-methoxyoxazolidine linker

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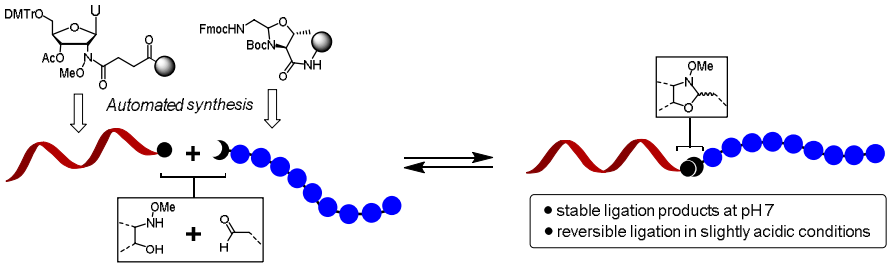
Supporting Information Placeholder



ABSTRACT: The formation of *N*-methoxyoxazolidines in the preparation of oligonucleotide-peptide conjugates was evaluated. The reaction occurred between unprotected 2'-*N*-(methoxy)amino-modified oligonucleotides and peptide aldehydes in reasonable yields when isolated. The reaction is reversible in slightly acidic conditions, and it is pH-responsive. The rate and the equilibrium constant may be varied with structurally different aldehydes, allowing an optimization of the ligation and cleavage rate of the resultant conjugates. Therefore, this concept can be considered a cleavable linker.

The interest to conjugate oligonucleotides (ONs) with peptides is currently undergoing a renaissance in order to development new tools for targeted delivery of ON therapeutics.[1] Of particular interest are peptides that in conjunction with large macromolecular delivery vehicles (e.g. nanoparticles, extracellular vesicles[2] and antibodies[3]) would recognize appropriate cell surface receptors and/or facilitate penetration of ONs through cellular barriers,[4,5] which, for example, is needed for endosomal escape.[6] As an example, antibodies are powerful tools to provide tissue-specific targeting of ONs,[7-9] but lead to endosomal entrapment of the ONs. In addition, membrane penetrating auxiliaries are needed, in which certain peptides may be utilized.[6-11] Similar needs may be required in nanoparticle-loaded ONs. While construction of these multifunctional constructs in the polydisperse form may be straightforward, synthesis of such uniform and covalently conjugated constructs is complex. In these scenarios you would need an efficient ligation to load ONs to antibodies or nanoparticles and then another orthogonal ligation, with nearly similar requirements, to conjugate ONs to peptides. Furthermore, cleavable linkers may be required to release the active ON cargo from the delivery vehicles.[12] Ideally the ligation chemistry itself would be reversible enough in the intracellular compartments but stable at physiological conditions. Therefore, eliminating the need of extra modifications in the structure to provide these characteristics. Examples of conventional linkages are: disulfide-[13] and hydrazone-ligation.[14,15] The first reaction is reversible in reducing conditions found in the cytoplasm and the latter in slightly acidic conditions, such as in endosomes (pH 5.5-6.2) and tumor tissues.[12] Both reactions are practically orthogonal allowing robust and efficient conjugation between appropriately functionalized unprotected ONs and peptides.

Despite the existing technologies, there is an obvious need for alternative conjugation chemistries that allow straightforward and orthogonal ligation between unprotected ONs and peptides. Plausible biodegradability of the linkage would be an additional benefit. Furthermore, it would be advantageous if the reactive moieties may be readily converted to a solid supported reagent. This would enable an automated synthesis of both reactive constituents (i.e. appropriately modified ONs and peptides). In order to meet the above mentioned needs and requirements, the present study describes a novel conjugation strategy, based on a pH responsive *N*-methoxyoxazolidine-formation between readily available 2'-*N*-(methoxy)amino-modified ONs and peptide aldehydes.

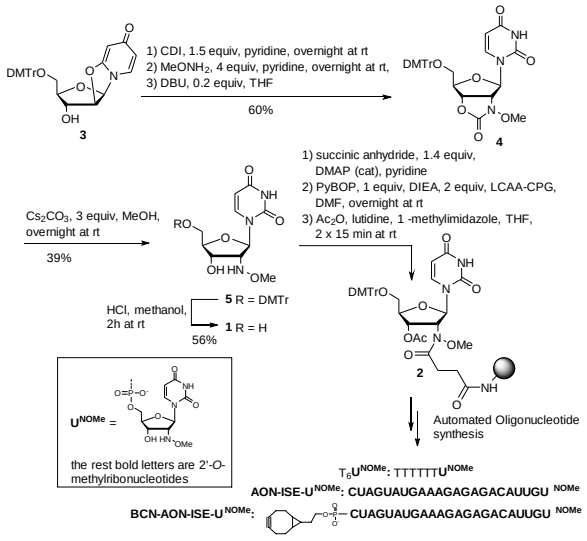
The rational for this design of ligation may be attributed to a combined chemistry of neoglycosides[16] and oxazolidines.[17-20] Neoglycosylation is an acid catalyzed reaction that occurs between sugar hemiacetals and *N*-methyl alkoxyamines.[16] It has been applied to the synthesis of various carbohydrate conjugates,[21-26] due to its orthogonality and good isosteric similarity to native glycan structures. However, the equilibrium constant of this reaction is relatively small (in aqueous solution < 100 L mol^{-1}, pH < 6).[27,28] By applying this same alkoxyamine-promoted acid catalyzed *O*,*N*-acetalization to form a 5-membered oxaozolidine ring,[17-20] the equilibrium constant may increase with the same favorable pH-profile of the reaction. To verify this hypothesis, the reaction with small molecular models was first studied. For this purpose, 2'-deoxy-2'-(*N*-methoxy-

amino)uridine (**1**) was synthesized from anhydrouridine **3** following the procedure by Ogawa et al.[29] (Scheme 1) and mixed with buffered solutions of different small molecular aldehydes (Table 1). The reaction rates and equilibrium constants were determined at pH 4, 5 and 6 using 5 mmol L^{-1} **1** and 5 mmol L^{-1} each aldehyde (at 22 ºC). As seen in Table 1, the reaction behaved like a neoglycosylation: the reaction was accelerated by lowering the pH from 6 to 4, but notably there was a relatively high equilibrium yield ($K = 0.4 – 9.0 \times 10^3$ L mol^{-1}). In each case, a dynamic equilibrium of (*R/S*)-stereoisomers of the oxazolidines was obtained (Figure 1). The reaction rate and equilibrium constant could be tuned by using different aldehydes. Relatively fast reaction rates were observed with aliphatic non-hindered aldehydes (acetaldehyde, butyraldehyde, $t_{0.5}$ ca 10 min. at pH 4.0). The highest equilibrium constant was observed with *N*-Bz-glycinaldehyde ($K = 9.0 \times 10^3$ L mol^{-1}, at pH 5.0). In our initial tests (data not shown) only trace of products was observed with ketones (e.g. acetone and levulinic acid) and reaction with conjugated aldehydes (benzaldehyde, *p*-methoxybentsaldehyde, pyridine-2-carboxaldehyde, cinnamaldehyde) was sluggish. Each product in Table 1 was also isolated, exposed to the same conditions, and the reverse reaction was studied. The decay rates with aliphatic aldehydes (acetaldehyde, butyraldehyde: $t_{0.5}$ decay ca. 3 – 5 h, 22 ºC) were comparable to hydrazones at pH 5.0[14,15], but the decays with benzyloxypropionaldehyde and *N*-Bz-glycinaldehyde were markedly slower. The formation obeyed a mixed reversible 2nd and 1st order rate law (cf. Equation S3). However, variation of the experimentally determined reaction rates extracted from the formation and decay was observed. This may be explained by partial obfuscating side reactions of the aldehydes.[30-32]

The reaction with *N*-Bz-glycinaldehyde was studied further to find optimal conditions to conjugate ONs with peptide aldehydes. Changing the solvent from H_2O to DMSO improved the equilibrium yield, as expected, but the reaction rate could be accelerated by mineral salts (NaCl, NaI, LiCl).[33] In optimized conditions (5.0 mM **1** + 7.5 mM *N*-Bz-glycinaldehyde, 1.5 equiv. in DMSO/AcOH/H_2O, 73:24:3, *v/v/v*, 2 M LiCl, 50 °C) the product was obtained in near quantitative yield (> 98%) in 15 min.

Solid support **2**, applicable to an automated synthesis of 2'-(*N*-methoxy)amino modified ONs, was next prepared (Scheme 1). 5´-*O*-(4,4´-dimethoxytrityl)-2´-deoxy-2´-(*N*-methoxyamino)uridine (**5**) was converted to a succinate and immobilized to a long-chain alkylamino-modified controlled pore glass (LCAA-CPG, loading of 21 µmol g^{-1}) using a PyBOP-promoted amide coupling. The unreacted amino groups and the 3'-OH group on the support were capped by acetic anhydride treatment. The Weinreb amide of **2** is cleavable by concentrated ammonia, which simplified the support preparation. To verify this prior to ON synthesis, a small aliquot of **2** was exposed to concentrated ammonia (5 h at 55 ºC), and the quantitative release of **5** was confirmed by LC-MS analysis. **2** was then used for the automated assembly of 2'-deoxy-2'-*N*-(methoxy)amino uridine (U^{NOMe}) extended ONs (Scheme 1): T_6U^{NOMe}, AON-ISE-U^{NOMe} (with the phosphorothioate backbone) and its bicyclononyne derivative BCN-AON-ISE-U^{NOMe} (with the native phosphodiester backbone). While T_6 is just a model, AON-ISE prevents expression of an androgenic receptor variant (AR-V7) as a potential therapeutic option for the treatment of castration-resistant prostate cancer.[34] The oligonucleotides were synthesized under standard conditions on 1.0 µmol scale using commercially available phosphoramidite building blocks (Scheme 1). 3-phenyl-1,2,4-dithiazoline-5-one (POS) was used as a sulfurization agent for AON-ISE-U^{NOMe}. Cleavage with concentrated ammonia, followed by RP HPLC purification (Figure S7) gave the desired 2'-*N*-methoxamino-modified oligonucleotides in isolated yields of 32% (T_5U^{NOMe}), 26% (AON-ISE-U^{NOMe}) and 17% (BCN-AON-ISE-U^{NOMe}). No premature reaction with aldehyde impurities was encountered during the purification (pH above 7). This is in contrast to what is typical for hydrazine-[35] or oxyamine-[36] modified oligonucleotides.

Scheme 1. Synthesis of CPG-immobilized 2´-deoxy-2´-(*N*-methoxyamino)uridine (2) and the 2'-deoxy-2'-(N-methoxyamine)-modified oligonucleotides



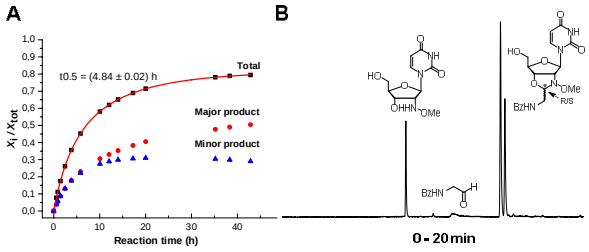
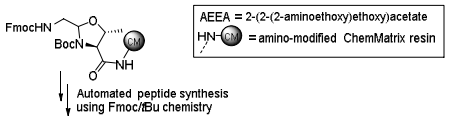


Figure 1. *N*-(Methoxy)oxazolidine formation between 1 and *N*-Bz-glycinaldehyde. A) Reaction profile of *N*-Bz-glycinaldehyde ligation at pH 4 (Table 1, entry 7) B) RP HPLC profile of the reaction at 35 h Cf. conditions in the Supporting Information.

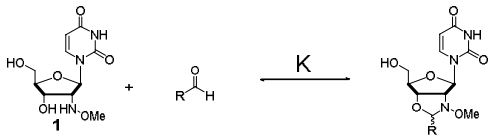
Scheme 2. Synthesis of peptide aldehydes for the conjugation of oligonucleotides



SpyTag-(AEEA)$_2$-Gly-H: H-Val-Pro-Thr-Ile-Val-Met-Val-Asp-Ala-Tyr-Lys-Pro-Thr-Lys-(AEEA)$_2$-Gly-H

THR-Gly-H: Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-Trp-Pro-Gly-H

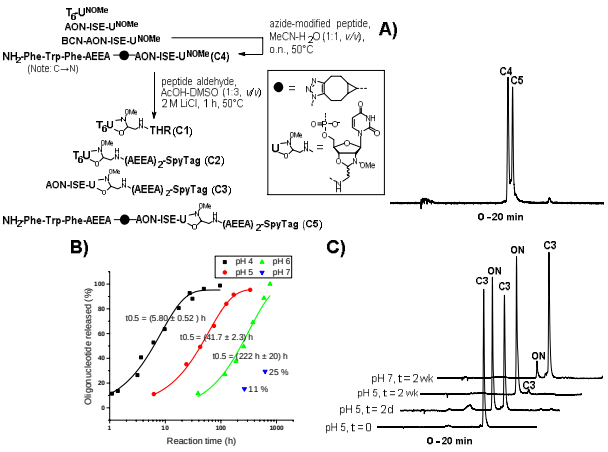
Table 1. Formation and decay of *N*-Methoxyoxazolidines between 2'-deoxy-2'-(*N*-methoxy)amino uridine and aldehydes



entry[a]	R	pH	t0.5 formation[b]	t0.5 decay[c]	equilibrium yield (%)	equilibrium constant, K (M^{-1})
1	Me	4	(7.90 ± 0.27) min	(1.54 ± 0.30) h	59	693 ± 29
2	"	5	(25.8 ± 0.6) min	(3.32 ± 0.40) h	62	856 ± 27
3	"	6	(3.93 ± 0.09) h	(43.1 ± 2.7) h	67	1211 ± 39
4	Pr	4	(11.8 ± 0.6) min	(1.50 ± 0.34) h	62	835 ± 49
5	"	5	(40.1 ± 0.6) min	(5.29 ± 0.58) h	54	499 ± 7
6	"	6	(4.14 ± 0.04) h	n/a	49	379 ± 3
7	$BzNHCH_2$	4	(4.84 ± 0.02) h	(16.1 ± 0.7) h	82	4958 ± 50
8	"	5	(33 h ± 1.3) h	(75.5 ± 16.3) h	86	9038 ± 1265
9	"	6	(12.8 ± 0.6) d	n/a	74	2224 ± 183
10	$BnOCH_2CH_2$	4	(21.6 ± 0.5) min	(2.00 ± 0.14) h	64	959 ± 28
11	"	5	(1.92 ± 0.05) h	(9.98 ± 0.38) h	66	1144 ± 39
12	"	6	(25.0 ± 0.7) h	(88.2 ± 6.8) h	63	939 ± 34

[a]22 °C, 50 mM buffer (cf. supporting information). [b]5 mM **1** and 5 mM aldehyde. [c]5 mM ligation product

Scheme 3. *N*-methoxyoxazolidine ligation of oligonucleotides to peptide aldehydes.



Notation: A) An example of RP HPLC profile of crude product mixture (**C5**), B) Oligonucleotide release profiles from the conjugate **C3** at pH 4-7. C) RP HPLC profiles of the cleavage. ON = AON-ISE-U^{NOMe} (cf. conditions in the supporting information)

For the synthesis of peptide aldehydes, a published protocol[37,38] was applied to immobilize freshly prepared Fmoc-Gly-H to L-threonine modified ChemMatrix-resin via an oxazolidine linkage (Scheme 2). This support and automatic Fmoc/tBu-chemisty were used to assemble two model peptides: SpyTag-$(AEEA)_2$-Gly-H and THR-Gly-H . SpyTag peptide, originally discovered by Zakeri et al,[39] binds irreversibly to its target protein (Spy-Catcher) forming an isopeptide bond between these peptide-protein components. This spontaneous and fast reaction has recently been found to have many applications in protein engineering. It may be applied for the development of modern biomaterials, though immunogenicity issues should be resolved prior to drug delivery applications.[40] THR binds to transferrin receptor,[41] and it has been applied to deliver RNA nanoparticles through blood brain barrier.[42,43] The peptides were cleaved from the support using common TFA–scavenger cocktails (cf. supporting information), precipitated in Et_2O and purified by RP HPLC to give the desired peptide aldehydes in 9% (SpyTag-$(AEEA)_2$-Gly-H) and 11% (THR-Gly-H) isolated yields.

Three ON-peptide conjugates (**C1**, **C2** and **C3**, Scheme 3) were prepared by mixing the 2'-(*N*-methoxy)amino modified ONs (4 mM T_6U^{NOMe} or AON-ISE-U^{NOMe}) with peptide aldehydes (8 mM, SpyTag-$(AEEA)_2$-Gly-H or THR-Gly-H, 2 equiv.) in a mixture of AcOH/DMSO 1:3, *v*/*v*, 2 M LiCl. The mixtures were incubated at 50 °C for 1 h, purified by RP HPLC (Figure S12) and lyophilized to dryness to give the desired ON-petide conjugates as white powders in 41% (**C1**), 12% (**C2**) and 28% (**C3**) isolated yields. No premature degradation of the *N*-methoxy oxazolidine-linkage occurred during HPLC purification or lyophilization. According to MS(ESI), no depurination or marked S→O conversion of the phosphorothioates[44] were detected in the ligation conditions. One bis-peptide-ON conjugate was also prepared by exposing BCN-AON-ISE-U^{NOMe} first to strain promoted azide-alkyne cyclo addition with an azide modified endosomal escaping peptide (N_3-AEEA-Phe-Trp-Phe-NH_2, to yield conjugate **C4**)[6], and then to *N*-methoxy oxazolidine formation (experimental details in the supporting information). A higher 10 equiv. excess of SpyTag-$(AEEA)_2$-Gly-H was used to give the desired bis-peptide-ON conjugate (**C5**) in 38% isolated yield (Scheme 3A).

Finally, deconjugation rates were studied by incubating the conjugate (**C3**, 10 µM) in a buffered solution at pH 4, 5, 6, and 7 and at 37 °C. HPLC and MS analysis of the released payload matched with the unconjugated AON-ISE-U^{NOMe} (but with partial desulfurization). Consistent with the small molecule model

studies (Table 1), **C3** was fully degraded in two weeks at pH 5 and four weeks at pH 6, whereas only 11% and 25% of the ON was released in two and four weeks (respectively) at pH 7 (Scheme 3B and 3C).

In conclusion, the reversible *N*-methoxyoxazolidine linkage was found to be a viable option for the preparation ON–peptide conjugates. This reaction occurred readily in slightly acidic conditions between 2'-*N*-(methoxy)amino-modified ONs and peptide aldehydes. The 2'-*N*-(methoxy)amino-modified ONs needed for the conjugation are readily available by using an automated synthesis and an appropriate solid support. Small molecule examinations demonstrated that the reaction favors aliphatic nonhindered aldehydes. The rate and the equilibrium constant of the reaction may be varied with structurally different aldehydes. The reaction is reversible and dynamic in slightly acidic conditions, whereas the products showed only marginal decay at pH 7. Therefore, the reaction showed on/off-characteristics. The decay rates proved slow, considering the idea of the cleavable linker, but the data derived from small molecule examinations suggest that there are other options for the peptide aldehydes, allowing an optimization of the cleavage rate, and the ligation itself.

ASSOCIATED CONTENT

Supporting Information

Experimental details and for the synthesis of **2**, 2'-*N*-methoxymodified oligonucleotides, peptide aldehydes and the conjugates **C1**-**C5**. Reaction profiles and characterization of the *N*-methoxy oxazolidines in Table 1.

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Author Contributions

The manuscript was written through contributions of all authors.

ACKNOWLEDGMENT

The financial support from the Academy of Finland (308931) and Business Finland (448/31/2018) is acknowledged.

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