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Efficient Functionalization of Oligonucleotides by New Achiral Nonnucleosidic Monomers

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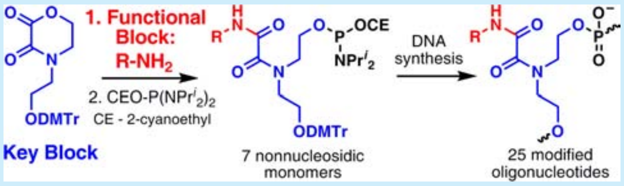
S Supporting Information

ABSTRACT: A novel synthetic strategy has been designed for preparation of achiral nonnucleosidic phosphoramidite monomers for automated solid-phase oligonucleotide synthesis. It is based on *O*-DMTr-protected 4-(2-hydroxyethyl)-morpholine-2,3-dione as the key compound and a family of building blocks obtained by its ring-opening by primary aliphatic amines. A series of nonnucleosidic phosphoramidites containing various side-chain functionalities was synthesized, and corresponding oligodeoxyribonucleotides incorporating modified units in single or multiple positions along the chain were prepared.

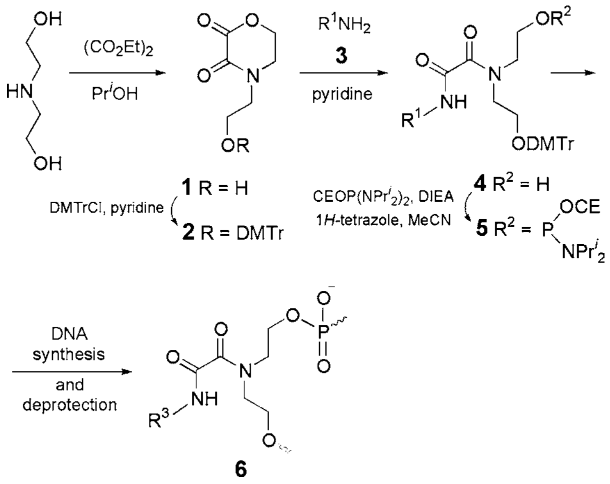


Oligonucleotides with pendant chemical functionalities are used widely as research tools for life sciences and as therapeutic or diagnostic agents.[1] Automated solid-phase synthesis by the 2-cyanoethyl phosphoramidite method is a general chemical method for preparing polynucleotides.[2] A majority of known chemical modifications could be conveniently tagged onto oligonucleotide sequences by solid-phase synthesis via either nucleosidic or nonnucleosidic phosphoramidites with tethered functional groups.[3] Nonnucleosidic monomers have an additional advantage of being independent of the oligonucleotide sequence since they are not usually involved in complementary binding. Sometimes a monomer is designed to mimick an acyclic nucleoside and thus maintains a five-atom distance between the P(V) atoms.[4] A majority of the available nonnucleosidic synthons could be split into two groups: racemic[5] and homochiral.[6] The former suffer from the disadvantage of producing diastereomeric oligonucleotides, which sometimes complicate isolation of the product.[7] The latter are normally derived from a homochiral natural precursor that is often costly.[5a,b] Frequently, preparation of a monomer bearing the required functionality may be accomplished only by multistep chemical synthesis in low yield.[8] Thus, the development of convenient, versatile, and efficient methods for preparation of nonnucleosidic phosphoramidites equipped with desired side-chain functionalities remains an important task.

We describe herein a novel strategy for the design of nonnucleosidic phosphoramidites for nucleic acid functionalization, which is a merger of two approaches: one utilizing the ring-opening of lactones by aliphatic amines[9] and another that uses activated oxalate esters for oligonucleotide derivatization.[10] Also, it takes into account our previous experience in the synthesis and application of oligonucleotides with nonnucleosidic inserts.[11]

We have applied our new building block strategy for the preparation of achiral phosphoramidites bearing various side-chains (Scheme 1, Table 1). The approach requires two separately functionalized building blocks. One is a key block that introduces the two hydroxyls needed for incorporation into DNA chain. Another is a functional block tagged with side-chain groups to convey specific properties to oligonucleotides. The key block is a common reagent for the preparation of a

Scheme 1. Synthesis of a Family of Achiral Nonnucleosidic Phosphoramidites and Oligonucleotides Thereof[a]



[a]Key: CE, 2-cyanoethyl; DIEA, *N*,*N*-diisopropylethylamine; DMTr, 4,4′-dimethoxytrityl.

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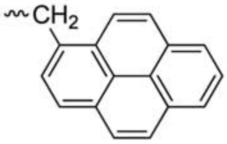
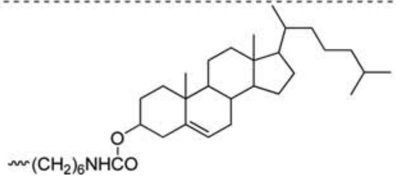
Table 1. Structure and Properties of Oligonucleotide Derivatives[a]

no.	sequence, 5' to 3'	monomer	modification **X** (**6**) $R^1 = R^3$	MALDI-TOF MS calc. [M]	found $[M+H]^+$	found $[M-H]^-$	RP-HPLC t_R, min
6.1	**X**GCGCCAAACA	**5a**	$\sim CH_2C{\equiv}CH$	3282.24	3284.75	–	7.6(A)[b]
6.2	GCGCC**XX**AAACA			3558.42	3560.76	–	7.7 (A)
6.3	**X**GCGCCAAACA	**5b**	$\sim (CH_2)_4OCH_2C{\equiv}CH$	3354.34	3355.79	–	8.4 (A)
6.4	GCGCC**X**AAACA			3354.34	3355.28	–	8.3 (A)
6.5	TTT**X**TTT	**5c**		2215.63	–	2213.72	13.2 (B)[c]
6.6	GCGCC**X**AAACA			3458.46	3460.56	–	7.2 (B)
6.7	**X**GCGCCAAACA			3458.46	3459.95	–	8.1 (B)
6.8	**XX**GCGCCAAACA			3910.86	3912.93	–	11.1 (B)
6.9	GCGCC**XX**AAACA			3910.86	3913.25	–	9.2 (B)
6.10	**X**GCGCC**X**AAACA			3910.86	3913.15	–	8.8 (B)
6.11	**X**TTTTTT	**5d**	$\sim (CH_2)_{11}Me$	2169.69	2170.05	2169.85	13.4 (B)
6.12	GCGCC**X**AAACA			3412.51	3413.68	3411.43	10.0 (B)
6.13	**X**GCGCCAAACA			3412.51	3414.41	3411.09	12.4 (B)
6.14	GCGCC**XX**AAACA			3818.97	3820.15	3816.99	14.2 (B)
6.15	**XXX**GCGCCAAACA			4225.44	4226.74	–	20.4 (B)
6.16	**X**TTTTTT	**5e**	$\sim (CH_2)_3NMe_2$	2086.51	2086.79	2084.92	8.6 (A)
6.17	**X**GCGCCAAACA			3329.34	3330.84	3325.63	14.3 (A)
6.18	GCGCC**X**AAACA			3329.34	3331.05	3325.64	13.5 (A)
6.19	A**XXX**GCGCCAAACA			4305.12	4306.67	–	13.5 (A)
6.20	**X**TTTTTT	**5f**	$\sim CH_2(CH_2CH_2O)_3(CH_2)_3NHY$ [d]	2204.65	2204.79	2203.38	9.1 (A)
6.21	TTT**X**TTTTTT			3117.24	3116.58	3115.38	9.2 (A)
6.22	TTT**XX**TTTTTT			3558.66	3557.85	3557.87	9.4 (A)
6.23	TTT**X**TTT	**5g**		2513.20	2512.75	2510.54	20.3 (B)
6.24	**X**TTTTTT			2513.20	2510.05	2511.44	22.6 (B)
6.25	TTT**X**TTTTTT			3425.80	3425.15	3425.25	17.4 (B)

[a] For experimental details, see the Supporting Information. [b] A: buffer B 50% MeCN. [c] B: buffer B 90% MeCN. [d] R^1: Y = CF_3CO, R^3: Y = H.

family of synthons with different side-chains. In each case oligonucleotide modification is accomplished by employing an appropriately functionalized monomer or combination of monomers during standard solid-phase synthesis.

We have selected diethanolamine as an inexpensive starting material that provides both hydroxyl groups required for incorporation into the oligonucleotide chain and a secondary amino group for attachment of a side-chain. *N*-Substituted diethanolamine derivatives[12] were described previously as precursors for nonnucleosidic phosphoramidites, but a difficulty of selective protection of only one of its two identical hydroxyls had significantly lowered yields of the products. We have solved this problem by simultaneous protection and activation of only one of the two hydroxyls as a cyclic oxalamide ester. The reaction of diethanolamine with inexpensive diethyl oxalate gives lactone **1** in high yield and purity.

It could be *O*-dimethoxytritylated in good yield affording DMTr-lactone **2** as our key block, which was used in all subsequent reactions with functional blocks. Those are primary aliphatic monoamines **3a**−**g**, which are either commercially available or are easily synthesized. The structure of the side-chain of a functional block (Table 1, R^1) defines specific property that is conveyed to a modified oligonucleotide: a bioconjugation site (**a**, **b**), a hydrophobic aromatic (**c**) or aliphatic (**d**, **g**) moiety, or a positive charge (**e**, **f**). Ring-opening of the key dimethoxytritylated lactone **2** by a functional primary amino block **3a**−**g** followed by phosphitylation yields a family of nonnucleosidic phosphoramidite monomers **5a**−**g** tagged with diverse side-chains (Scheme 1). The monomers were successfully tested in solid-phase oligonucleotide synthesis according to standard phosphoramidite protocol, except that a higher concentration of 0.15 M and longer coupling time of 7 min were used. We were able to introduce up to three consecutive modifications into any position of choice within oligonucleotide chain in good overall yield.[13]

The copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC)[14] has found widespread use for bioconjugation as a widely popular example of click chemistry.[15] A number of methods for preparation of alkyne derivatives of oligonucleotides were published.[16] However, a reliable method for side-chain alkynyl group attachment in the middle of the chain would still be useful.[17] We have tagged an alkyne onto the side-chain of a nonnucleosidic monomer via either a shorter (**5a**) or longer five-atom (**5b**) linker. Both monomers were successfully applied to make alkynyl oligonucleotides by solid-phase synthesis. The potential of the alkyne derivatives for click chemistry was demonstrated by facile coupling of an azido Cy3 dye to an alkynyl oligonucleotide under CuAAC conditions (see the Supporting Information).

Pyrene is a robust fluorophore that has been widely used to sense subtle changes in nucleic acid geometry.[18] We have attached pyrene via its aminomethyl derivative to the side-chain of a nonnucleosidic monomer **5d**. The reagent was successfully used for solid-phase synthesis of oligonucleotides labeled with one or more pyrene residues in various positions. When one or two pyrenyl groups were attached to the 5′-end, a marked stabilization of complementary complex resulted, which correlates well with previous observations.[19]

Poor cell uptake is a problem that currently limits therapeutic application of oligonucleotide and their analogues.[20] Functionalization with lipids is known to increase intracellular delivery of oligonucleotides.[21] We have designed a nonnucleosidic monomer **5d** for incorporation of dodecyl groups into synthetic oligonucleotides at one or several positions within their chain. It has resulted in a marked rise in lipophilicity of the oligonucleotides with the increase of the number of dodecyl groups (Figure 1).

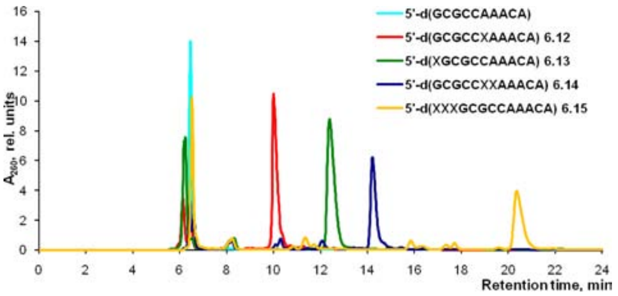


Figure 1. RP-HPLC traces of oligonucleotides functionalized with one, two, or three dodecyl groups; R^3 = dodecyl, X marks the position of the modification.

Nucleic acids are polyanions and as such they do not readily cross cell membranes. Introduction of positively charged groups into oligonucleotides may improve their cell uptake as well as result in better target binding due to partial neutralization of negatively charged phosphate groups. We have designed two nonnucleosidic monomers incorporating aliphatic amino groups that should be positively charged under physiological conditions. One (**5e**) has a tertiary dimethylaminopropyl group, and another (**5f**) has a primary amino group on a 13-atom hydrophilic poly(ethylene glycol) linker. In addition, the latter reagent could potentially be employed for label attachment to, or bioconjugation of, modified oligonucleotides via the primary amino group.

It was shown that cholesterol attachment can improve cellular permeability of oligonucleotides and their analogues.[22] The property is especially important for therapeutic antisense agents. We have applied our new method for cholesterol attachment via aminohexyl carbamate side-chain. The monomer **5g** proved to be sparingly soluble in acetonitrile, so 1:1 mixture of acetonitrile with THF was used, which resulted in smooth incorporation of the cholesterol group into an oligonucleotide chain in good yield.

In conclusion, we have described a novel, versatile and efficient strategy for the design and synthesis of a family of side-chain functionalized nonnucleosidic phosphoramidite monomers for automated solid-phase oligonucleotide assembly. The strategy is based on a simple protection-activation reaction of diethanolamine to produce a key lactone that could be ring-opened by a series of primary aliphatic amines. The monomers were successfully employed to synthesize oligonucleotides functionalized by one or more pendant groups that convey hydrophobicity, positive charge, or click chemistry potential. The new chemistry relies on simple, inexpensive, and easily obtainable starting materials and good-yielding convergent chemical syntheses. It should be readily applicable to a range of backbone modifications such as RNA or other biologically important 2′-substituted analogues. Our method could be conveniently used to obtain oligonucleotides with diverse sets of properties useful for design of new diagnostic nucleic acid probes and potential antisense therapeutic agents with improved cell delivery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, NMR spectra for monomers and precursors, HPLC, PAGE and MALDI-TOF data for oligonucleotides, and thermal denaturation for pyrene-labeled complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(13) Oligonucleotides with internal modifications or 5′-modified with DMTr group on are stable under normal conditions of final deprotection (conc aq ammonia, 55 °C, 12 h). Specific conditions are required for basic cleavage of the oxalamide linker (see refs 12a and 17). We have not observed any particular difference in stability of oxalamide-modified and unmodified oligonucleotides under various conditions such as RP-HPLC, PAGE, thermal denaturation, or click conjugation.

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