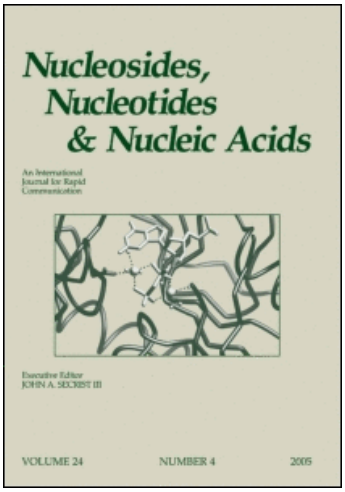

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Concept of "Binary Oligonucleotide Reagent"

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CONCEPT OF "BINARY OLIGONUCLEOTIDE REAGENT"

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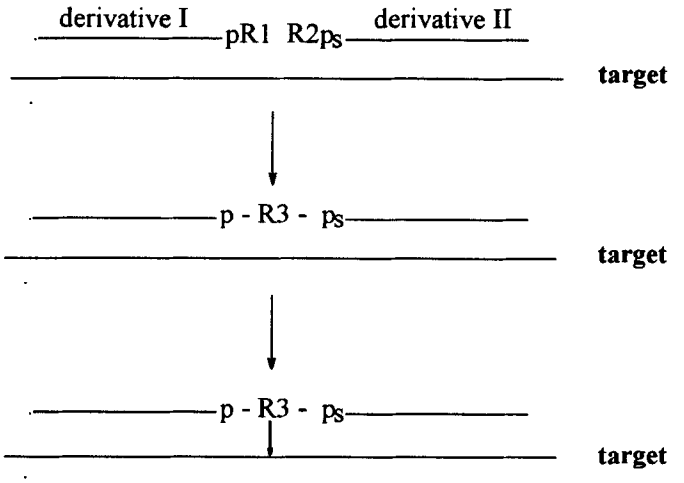
ABSTRACT: A new strategy for site-directed chemical modification of NA is described. NA-target-driven autoligation reaction between two oligonucleotide derivatives with N-(2-chloroethyl)-N-(p-formylphenyl)-N-propyl-N-3-ydeneamino and 4-carbohydrazide-phenyl groups at their opposing termini results in the NA-target modification, which is several times more effective than modification by one of the derivatives.

One of the generations of antisense oligonucleotide analogs is represented by oligonucleotide derivatives bearing cross-linking, cut off, and intercalating groups [1]. Shaw et al. [2] and Cowart et al. [3] describe cross-linking oligonucleotide derivatives self-activated as the result of forming a perfect complementary complex with the NA target (hybridization triggered derivatives). Lynn [4], Letsinger [5,6], and Gryaznov [7] describe another hybridization-triggered process - autoligation reactions.

Here an attempt is made to combine the advantages of the two hybridization triggered processes into one approach. Two oligonucleotide derivatives used in this approach are named "binary oligonucleotide reagent" (BOR).

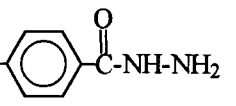
BOR consists of two oligonucleotide derivatives, I and II, the oligonucleotide parts of which are complementary to neighbouring sites of an NA-target. Derivative I bears a reactive group R1 at its 3'-end, derivative II bears R2 at the 5'-end. These derivatives do not react with each other when in a solution. When they are in a perfect complementary complex with the NA-target, a new group R3, more reactive than R1 and R2, is formed as the result of a reaction between R1 and R2 and their linking. Then R3 modifies the NA-target.

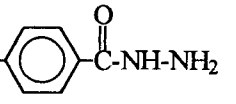


Scheme I

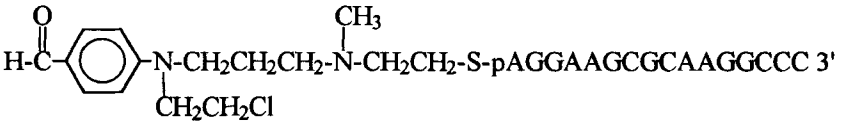
For demonstration of this approach in vitro the following components were used:

Derivative I:

a) 5' GGTAGAGCGCTTACCTp -NH-CH_2-  -C-NH-NH_2

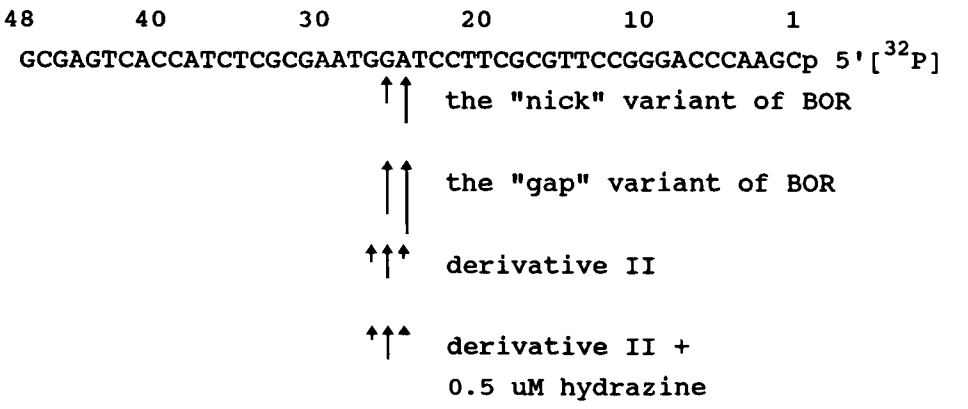
b) 5' TGGTAGAGCGCTTACCp -NH-CH_2-  -C-NH-NH_2

Derivative II:



Scheme II

Target. A 48-mer polynucleotide:



Derivatives Ia and Ib were synthesized from corresponding 3'-phosphate of the oligonucleotides and 4-carbohydrazidebenzylamine [8] using a commonly used condensation reaction [9] by a method similar to [10]. The products were isolated by ion-exchange HPLC on a Lichrosphere-NH_2 column [11] and characterized by electrophoresis in 20% denaturating PAAG following 5'-[^{32}P] labelling by T4 polynucleotide kinase and reaction with p-nitrobenzaldehyde [12] (Fig. 1).

Derivative II was synthesized according to [13]. The oligonucleotide was converted into the 5'-phosphorothioate by T4 polynucleotide kinase enzymatic reaction with ATPgammaS [13]. The 5'-phosphorothioate of the oligonucleotide was alkylated with N-methyl-N,N'-di(2-chloroethyl)-N'-(p-formylphenyl)propylenediamine 1,3 [14], and the product was isolated by HPLC [11].

Derivatives Ia, Ib, and II were desalted by gel filtration and concentrated to an appropriate concentration on a Speed Vac Concentrator before using in subsequent reactions.

The reaction mixtures contained: 0.4 uM [^{32}P]-target; 1 uM derivative Ia (Ib); 1 uM derivative II; 10 mM HEPES-KOH, pH 7.3. In the control experiments the corresponding precursors of derivatives I and II were used at 1 uM concentration. After 60 hours incubation at 37° the products were analyzed by electrophoresis in 20% denaturating PAAG. (Fig. 2a).

In preliminary experiments under the same conditions, when 1 uM concentrations of derivative Ia (Ib) and derivative II were used, the product of their ligation (32-mer imine

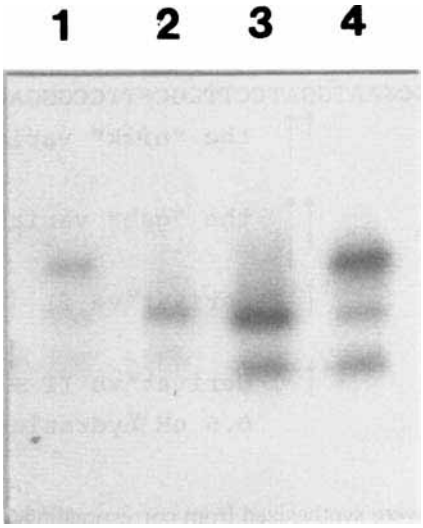


FIGURE 1. Radioautograph of the gel. Derivative Ia: 1 - product + pNBA; 2 - product. Derivative Ib: 3 - product; 4 - product + pNBA.

derivative) was detected only in the presence of the target (0.4 uM). A typical picture is presented in Fig.2b [15].

The major [^{32}P]-products of the target modification were cut off from the gel and counted with a scintillation counter. The yields of BOR modifications (evaluated by conversion of the target to the modification product) were 10%. The yields of the modified target in reactions with derivatives I or II alone did not exceed 3.0% and 1.5% respectively. In other control experiments, when one of the BOR components was replaced with its oligonucleotide precursor, the yields of the modified target did not exceed 3.6%. The yield of modification of this target by sodium borohydride activated derivatives II was 20% [16]. When the target modification by BOR was made at 20° and 10° during 168 hours and 240 hours respectively, at ten times higher concentrations of the components, the difference in the yields between modification by BOR and by its individual components was not so pronounced.

Several control experiments were also made on the stability of der.Ia (Ib) and the 32-mer imine derivative under the conditions of BOR modification (37°, 60 hours). No more than 60% of der.Ia (Ib) were lost therewith. The 32-mer imine derivative decomposed approximately for 80% in the absence of the target, but was stable with it.

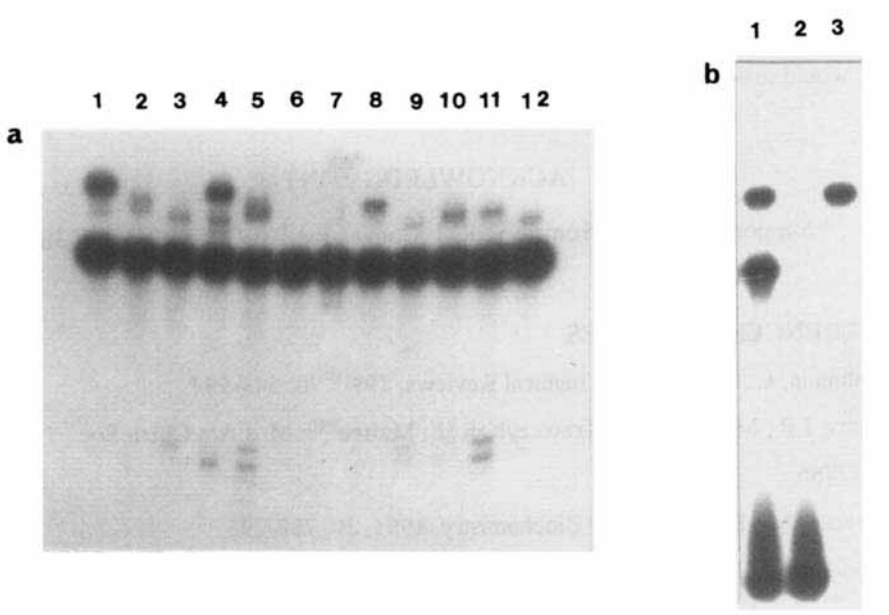


FIGURE 2a. Radioautograph of the gel.
1 - der.Ia + der.II (BOR, nick variant); 2 - der.Ia; 3 - der.II; 4 - der.Ib + der.II (BOR, gap variant); 5 - der.Ib; 6 - [^{32}P]-target alone; 7 - der.Ia + p$_s$AGGAAGCGCAAGGCCC (precursor of der.II); 8 - der.Ia + AGGAAGCGCAAGGCCC (starting material); 9 - GGTAGAGCGCTTACCTp (precursor of der.Ia) + der.II; 10 - der.Ib + p$_s$AGGAAGCGCAAGGCCC (precursor of der.II); 11 - der.Ib + AGGAAGCGCAAGGCCC (starting material); 12 - GGTAGAGCGCTTACCp (precursor of der.Ib) + der.II.

FIGURE 2b. Radioautograph of the gel.
1 - [^{32}P]-der.Ib + der.II + [^{32}P]-target; 2 - der.Ib + der.II; 3 - [^{32}P]-target alone. The specific radioactivity of der.Ib was twice as high as that of the target.

The products of BOR modifications were treated according to the Gilbert sequencing procedure with 10% piperidine. The results of their cleavage are represented in Scheme II with arrows. They indicate that we achieved a sequence-specific chemical modification of the target by BOR in both variants.

The modification by BOR is more effective than modification with any of the components alone. It may be suggested that the proposed strategy has some potential advantages as compared with those existing [1]. Development of better chemical groups R1 and R2, more effective in target modification reactions, that would be more selective to

each other and, as a result, less prone to side reactions at the cell surface and inside the cells, would make the BOR approach promising for in vivo systems.

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