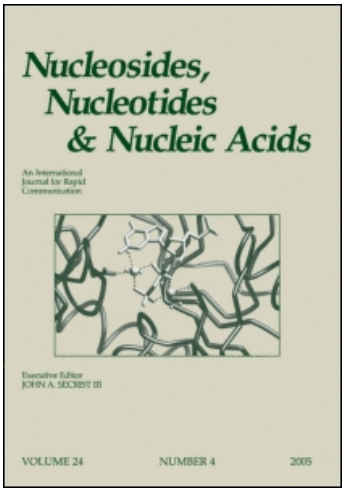

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Specific Intrachain Introduction of Reporter Groups into Oligonucleotides as Substituents at Internucleotidic Linkages

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SPECIFIC INTRACHAIN INTRODUCTION OF REPORTER GROUPS INTO OLIGONUCLEOTIDES AS SUBSTITUENTS AT INTERNUCLEOTIDIC LINKAGES

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Abstract: Two routes to the introduction of biotin labels into oligonucleotides via an intrachain phosphotriester linkage are described. A "loop linker" was prepared on this basis for attachment of ds DNA to an avidin-coated solid phase.

INTRODUCTION

The use of oligonucleotides as medicinal probes requires the introduction of a label for detection of hybridization. As an alternative to radioisotope labelling, which is not practical for clinical routine, non-radioactive reporter groups have been introduced either at the nucleobases or at the end of the oligonucleotide chain via spacer units. While base attachment a priori impairs hybridization, a destabilizing effect has also been observed from biotin labels attached via spacers to the chain end of probes[1]. Therefore, we have explored, as a third alternative, the intrachain biotin attachment via a phosphotriester internucleotidic linkage[2].

RESULTS AND DISCUSSION

A 2-aminoethyl spacer substitutent was introduced at an internucleotidic linkage by two routes: 1. via a preformed dinucleotide block. As an example, 5'-O-dimethoxytrityl-thymidine was converted to the o-chlorophenylphosphorotriazolide[3] and further reacted with unprotected thymidine[4] (yield: 55 % of dinucleoside-(o-chlorophenyl-)phosphate). Deprotection with NH_3/pyridine (3:1) (2d, r.t.) to dinucleoside phosphate (I), then reaction with 9-fluorenylmethoxy-

carbonyl-aminoethanol/mesitylene sulfonyl nitrotriazolide/ N-methylimidazole, gave 50 % dinucleoside-(Fmoc-aminoethyl-) phosphate (II) (FAB-mass spectrum: m/z=1285-1287 $(M^-, (M-H)^-, (-2H)^-)$; 1309 $(M+Na)^-$; ^{31}P-NMR in $CDCl_3$: δ = -4.34;-4.59ppm), which was converted into 3'(ß-cyanoethoxy-)N,N-diisopropyl-amino-phosphoramidite (III; yield: 82 %). 2. 5'-O-dimethoxy-tritylthymidine was converted into 3'-bis(N,N-diisopropyl-amino-)phosphoramidite[5], then, without isolation of intermediate, further reacted with 9-fluorenylmethoxycarbonyl aminoethanol to yield, after column chromatography, 20 % IV (^{31}P-NMR in $CDCl_3$: 145.80; 146.68 ppm). Oligonucleotide syntheses were done in an automated synthesizer using either III or IV, the yields being ca. 87 % for cycles with III, ca. 99 % with IV. The Fmoc group was removed by morpholine

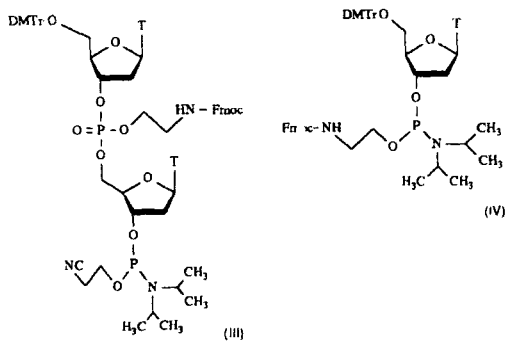

FIG. 1

in ethanol (60 min., r.t.) without release from the polymer support[7]. The oligomer was deprotected - phenoxyacetyl from dA and dG[6], acetyl from dC and ß-cyanoethyl from phosphate - and released from the support by ammonia treatment (60 min., r.t.). After further purification, the biotinylation was done in up to 90 % yield by treatment with 7-fold N-biotinyl-succinimide in DMF: 0.01 M potassium phosphate buffer (pH7.5) = 1:1 (12 h, r.t.). Detection of the biotin substituent was done by FAB-MS (DMTrdTp(OCH_2CH_2NH-biotin)TLev: m/z = 1313 $(M+Na)^+$; $(M-H)^-$; Lev = levulinyl-), N,N-dimethylamino-

cinnamic aldehyde spray and affinity towards streptavidin.

Hybridization Behaviour: Preliminary hybridization studies were conducted in parallel with decathymidylate sequences a) internally biotinylated by insertion of IV, b) end-labelled by the "amino-link" technique (Applied Biosystems), c) without marker sequence. The T_m values of hybridization to dA_{10} in these experiments were within the precision of measuring identical with the T_m value for hybridization of the unmodified decathymidylate (m.p. 18.0-18.5°C). This is in accordance with findings of Fidanza and McLaughlin[8], who, parallel to our work studied the melting behaviour of internally spin-labelled oligonucleotides. The spin labels, in this case, were introduced by reaction of a phosphorothioate internucleoside linkage with spacered label molecules. Our approach is more versatile, in that the oligonucleotide is preformed with a multipurpose backbone spacer, to which not only biotin, but a variety of reporter groups can be attached. Further studies on the comparison of probes labelled according to a) and b) in in vitro tests and in situ hybridization are under way.

Preparation of a Biotinylated "Loop Linker": As an application of intrachain labelling we constructed a multipurpose biotinylated "loop linker" (V) for attachment of double-stranded DNA fragments to solid phases. The sequence of V is shown in Fig. 2. The synthesis was done using IV in 99 %

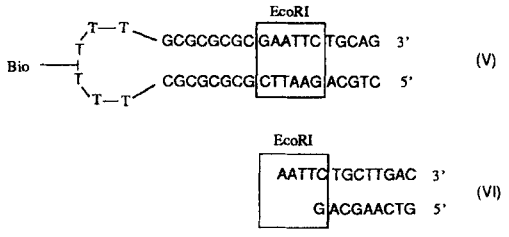

FIG. 2

average yield per cycle. The yield of purified V was 2.7 OD_{260}. Treatment of V bound to Streptavidin-Agarose (capacity: 120 pmol/ml) with EcoRI exposed 42 % of sticky ends, to which a labelled synthetic DNA fragment (VI) was attached by DNA ligase (efficiency 53 %, as monitored by ^{32}P-labelling of VI). Further applications of this and similarly designed "linker loops", which extend the scope of the previously described "splinker" principle[9] to enzymatic solid-phase polynucleotide reactions[10], are under investigation.

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