Site specific functionalization of oligonucleotides for attaching two different reporter groups

Sudhir Agrawal* and Paul C.Zamecnik
Worcester Foundation for Experimental Biology, Maple Avenue, Shrewsbury, MA 01545, USA

Received June 25, 1990; Revised and Accepted August 14, 1990

ABSTRACT

The synthesis of an oligonucleotide functionalized to attach two different reporter groups at specific internucleotide linkages is described. To incorporate the amine specific reporter group the internucleotide linkage is modified to phosphoramidate (N-1-aminoalkyl) and for a thiol specific reporter group the internucleotide linkage is modified to a phosphorothioate diester. The synthetic cycle for introducing the modified internucleotide linkages at specific sites can be carried out using an automated DNA synthesizer. Combination of reporter groups have been attached successfully.

INTRODUCTION

In both basic and applied molecular biology, there is widespread use of synthetic oligodeoxyribonucleotides carrying a reporter group. In addition to their use as hybridization probes (1), they have been used for diagnostic procedures (2), automated sequencing (3–6), electron microscopy (7), fluorescence microscopy (8,9), x-ray crystallography (10), hybridization affinity chromatography (11,12) and probing of nucleic acid structure (13–15). Recently, oligonucleotides covalently attached to intercalators, chain cleavers or alkylating agents have been used to regulate gene expression (16,17). We were interested in preparing oligonucleotides to which two different reporter groups can be attached at specific sites for studying protein-nucleic acid interaction and localization of target nucleic acid sequences by use of non-radiative fluorescence resonance energy transfer (FRET) (14,18). Similarly, facile attachment of two different chemical moities to an oligonucleotide, such as an intercalator to stabilize the duplex stability and a separate affinity reagent capable of bringing about chemical modification of the target sequence, adds an additional dimension to the design of oligonucleotides as inhibitors of gene expression.

To date, in general, oligonucleotides have been functionalized to incorporate single or multiple molecules of the same reporter group. The functionalization of oligonucleotides for attaching these reporter groups has largely relied upon, (a) functionalization of 5′- or 3′- termini by numerous chemical reactions using deprotection oligonucleotides in aqueous or largely aqueous medium (19–24), (b) synthesizing modified nucleosides containing a masked primary amine group on the base heterocycle and its incorporation into oligonucleotides during synthesis (7,10,25–29) and, (c) use of suitably protected chemical moieties, which can be coupled at the 5′-terminus of protected oligonucleotide during synthesis (30–35).

The other possible route to attach a reporter group at specific sites in an oligonucleotide is at a suitably functionalized phosphate backbone. Phosphoramidate analogues of dinucleotides have been prepared and attached to various ligands (36–38). Recently phosphorothioate diester internucleoside linkages have been incorporated at specific sites in oligonucleotides (39,40,41) and thiol specific reporter groups have been attached (40,41).

Here we report site specific functionalization of oligonucleotides with an -SH group and a -NH_2 group for attaching two different reporter groups–one thiol specific and the other amine specific (Figure 1). A preliminary communication of a study of incorporating aliphatic amines at internucleoside phosphates as phosphoramidates to attach nonradioactive labels has been published (42).

METHODS AND MATERIALS

Oligonucleotide synthesis was carried out using either phosphoramidite (43) or H-phosphonate (44,45) chemistry and also a combination of both using an automated DNA synthesizer (Milligen-Biosearch 8700, Burlington, MA). Nucleoside β-cyanoethylphosphoramidite and nucleoside H-phosphonate were obtained from Milligen. N-1-trifluoroacetylhexanediamine was prepared as published earlier (42). The rest of the chemicals were purchased from the sources mentioned in brackets: monobromobimane (Calbiochem); Fluorescein isothiocyanate (Sigma); NHS-ester of biotin (Clonetech); 5-(2-(iodoacetyl)-amino)ethyl) aminonapthalene-1-sulfonic acid [1,5-I-AEDANS] and 5-idoacetamidofluorescein (Molecular Probes).

HPLC was carried out as previously described (30,42). All the oligonucleotides synthesized for the present study are listed in Table 1. Oligonucleotide 1 was assembed using standard phosphoramidite chemistry.

Oligonucleotide 2, carrying one phosphorothioate internucleoside linkage at the 5′-terminus was assembled using a combination of both phosphoramidite and H-phosphonate chemistry (39).

For assembling oligonucleotide 3, the first coupling was carried out using H-phosphonate chemistry, followed by oxidation with

* To whom correspondence should be addressed

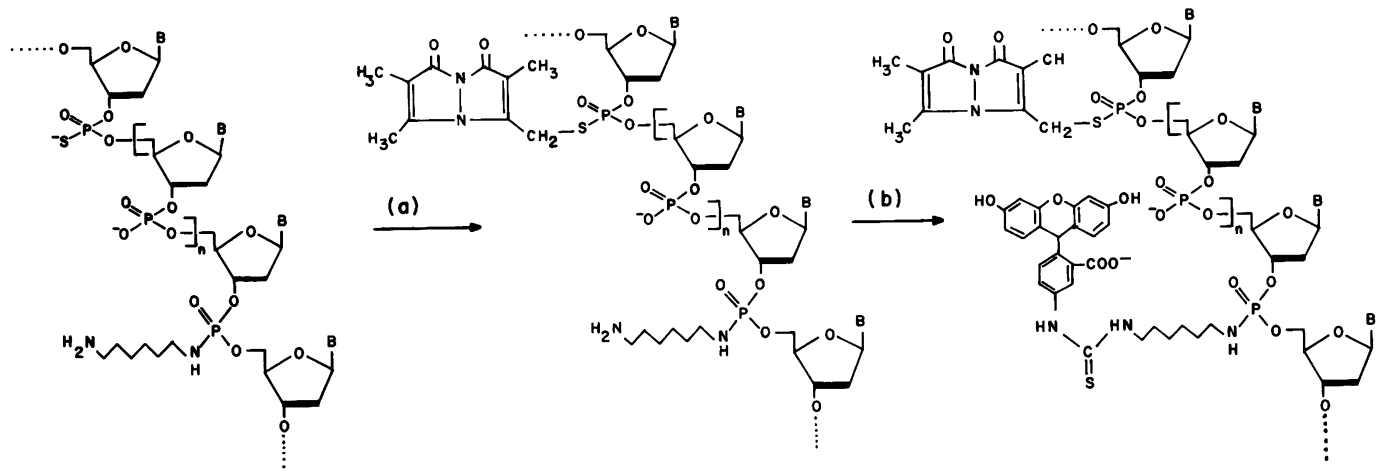

Figure 1. Schemes for synthesis and attachment of two different labels to an oligonucleotide. (A) Oligonucleotide carrying one phosphorothioate diester and one phosphoramidate (aminohexyl) internucleoside linkage at specific sites. The oligonucleotide (A) can be reacted with thiol-specific nonradioactive labels, in here monobromobimane, which attaches only to the phosphorothioate diester linkage, yielding a phosphorothioate triester (B). Under the conditions for attaching thiol specific labels, the amino group is unreactive. Oligonucleotide (C) is the reaction product of fluoroscein isothiocyanate and (B).

N-1-trifluoroacetylhexanediamine (42). The next fourteen couplings were carried out using phosphoramidite chemistry, using standard conditions (oxidation with iodine after every coupling). The last coupling was then carried out using H-phosphonate chemistry, followed by oxidation with sulphur in triethylamine-carbon disulfide-pyridine to generate one phosphorothioate internucleoside linkage.

Reaction of Monobromobimane with Oligonucleotide 2

4 A_{260} units of oligonucleotide 2 was dissolved in 250 µl of 0.25 M Tris/HCl buffer (pH-7.2), mixed with 250 µl of 10 mM monobromobimane in acetonitrile and the mixture left at room temperature for 6 hours. Analytical reversed phase HPLC showed greater than 90% conversion to a more retained peak. The excess monobromobimane was removed by gel filtration on Sephadex G-25 and then purified on reversed phase HPLC (figure 2b, retention time 23.02, 23.92 min.), to yield 3.4 A_{260} units (85% yield) of oligonucleotide 4.

Reaction of Monobromobimane with Oligonucleotide 3

Four A_{260} units of oligonucleotide 5 were reacted with monobromobimane using the same condition as for oligonucleotide 2. The product peak, oligonucleotide 5 was retained more than oligonucleotide 3 on a reversed phase column (figure 4b, retention time 25.20, 25.95 minutes, in a ratio of 1:1), yield 3.3 A_{260} unit (82.5%). UV-VIS spectrum was recorded in water λmax 260, 385 nm; fluorescence spectrum—Ex. 394 nm, Em. 480 nm.

Reaction of Oligonucleotide 5 with NHS-ester of Biotin

One A_{260} unit of oligonucleotide 5 was reacted with NHS-biotin (30). After 60 minutes of incubation, reversed phase HPLC showed a major peak more retained than oligonucleotide 5 (figure 4c, retention time 31.22, 31.63 min.), yielding 0.75 A_{260} unit (75%) of oligonucleotide 7. UV-VIS spectrum, λmax 260, 385 nm; fluorescence spectrum, Ex. 397, Em. 480 nm.

Reaction of Oligonucleotide 5 with Fluorescein Isothiocyanate

To 1.5 A_{260} units of oligonucleotide 5 in 200 µl water was reacted with fluorescein isothiocyanate as previously described (30). The mixture was incubated at 37°C in the dark for 2 hours and the product examined by reverse phase HPLC (figure 4d, retention time 30.79, 31.6 min). During this time, the reaction had proceeded to greater than 90%. The multiple peak was collected and desalted on Sephadex G-25 yielding 0.9 A_{260} units (60%) of oligonucleotide 7. UV-VIS spectrum, λmax 260, 385 and 470 nm; fluorescence spectrum—Ex. 394 nm, Em. 460 and 517 nm.

TABLE I Oligonucleotides Synthesized.

Oligo-nucleotide	
1.	GpTpApApApApCpGpApCpGpGpGpApCpCpApGpT
2.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApGpT
3.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApG$_p{}_n$T
4.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApGpT Bimane
5.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApG$_p{}_n$T Bimane
6.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApG$_p{}_n$T Bimane Biotin
7.	$G_p{}_s$TpApApApApCpGpApCpGpGpGpApCpCpApG$_p{}_n$T Bimane Fluorescein

p = phosphate p_s = phosphorothioate p_n = phosphoramidate

RESULTS AND DISCUSSION

In peptides, two different nonradioactive labels have been attached, either to the thiol group of cysteine, to the amine group of lysine, or to carboxyl terminus (45,46). Derivatives of nonradioactive labels such as maleimide, haloacetyl, aziridinyl sulfonamides react efficiently with a thiol group at physiological pH range, whereas under these conditions an amine group is protonated and remains unreactive. Derivatives of nonradioactive labels such as isothiocyanate, N-hydroxysuccinimide, sulfonyl halides, anhydride, aldehyde and glyoxal have high reactivity with an amine group and react best above pH 8. At this pH, the amine group is unprotonated and reactive.

To demonstrate the functionalization of oligonucleotides with -SH and -NH_2 group for attaching two different reporter groups, the synthesis of the oligonucleotide sequence, 5'- GTAAAA-CGACGGCCAGT was carried out. This oligonucleotide contains the 'universal' DNA sequencing primer for M13 templates (30). Oligonucleotide 1 was synthesized as a control.

To study the functionalization of an oligonucleotide for attaching a reporter group designed to react with a thiol group, oligonucleotide 2 was assembled. This oligonucleotide contains a single phosphorothioate diester internucleoside linkage at the 5'-terminus. Oligonucleotide 2 was assembled by using both nucleoside β-cyano-ethyl phosphoramidite as well as nucleoside H-phosphonate. The first fifteen couplings were carried out using phosphoramidite chemistry and standard conditions (oxidation with iodine). To the same CPG bound 16-mer, the last coupling was carried out using H-phosphonate chemistry. The 17-mer containing one unoxidised H-phosphonate internucleoside linkage was oxidised with sulfur to generate a phosphorothioate diester linkage (figure 3). On ion exchange HPLC, the elution time of oligonucleotide 2 was slightly longer than that of the corresponding oligonucleotide 1, without one phosphorothioate diester linkage. On reversed phase HPLC, oligonucleotide 2 (ion exchange purified) gave a doublet peak, resulting from the two phosphorus diasterioisomers (Rp and Sp) at the phosphorothioate diester internucleoside linkage (figure 2).

Reaction of oligonucleotide 2 with monobromobimane at physiological pH and room temperature yielded oligonucleotide 4 almost quantitatively, as monitored by reversed phase HPLC. Reaction with 1,5-I-AEDANS and also with 5-iodoaceta-midofluorescein also gave similar results. It was important to study the hydrolytic stability of the phosphorothioate triester in oligonucleotide 4, under the condition for attaching labels to an amino group, before an attempt was made to synthesize an oligonucleotide which is functionalized with both -SH as well as -NH_2 groups. Oligonucleotide 4 incubated with pH 9 buffer was analysed by reversed phase HPLC. After 24 hours, the phosphorothioate triester linkage in oligonucleotide 4 was completely hydrolyzed to a mixture of 16-mer and 17-mer. Hydrolysis of the phosphorothioate triester proceeded by desulfurization and chain cleavage. The rate of hydrolysis is dependent on the reporter group, and also in some cases hydrolysis was proceeded by desulfurization only and minor chain cleavage was noticed. At pH 9, in 4 hours at 37°C, the hydrolysis of phosphorothioate triester was only 5−10%, which is the optimal condition to attach most of the labels to the amino group.

Oligonucleotide 3 was assembled to demonstrate attachment of two different reporter groups. First, coupling was carried out using H-phosphonate chemistry. The CPG-bound dinucleoside H-phosphonate was then oxidized with N-1-trifluoro-acetylhexanediamine in carbon tetrachloride, to generate a phosphoramidate linkage (42). This phosphoramidate linkage contains a suitably protected amine group, which remains masked under oligonucleotide synthesis either by phosphoramidite or H-phosphonate chemistry. Next, fourteen couplings were carried out using phosphoramidite chemistry and standard oxidation steps with iodine. The last coupling was then carried out using H-phosphonate chemistry, followed by oxidation of the H-phosphonate internucleoside linkage with sulfur to generate a phosphorothioate diester linkage. Deprotection of oligonucleotide 3 was carried out using standard condition. The product was

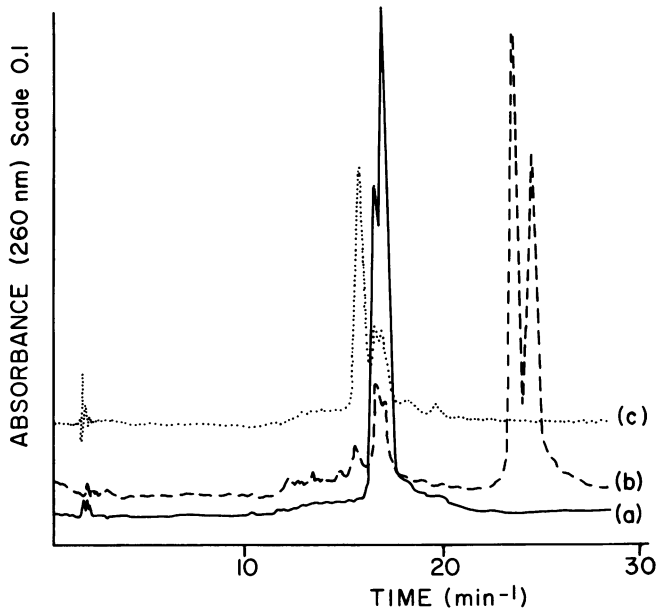

Figure 2. Reversed phase HPLC shows traces of (A) oligonucleotide 2, (B) oligonucleotide 4 and (C) oligonucleotide 4 after incubation with pH9 buffer for 24 hours. It is to note that hydrolysis of oligonucleotide 4 proceeds by desulfurization and chain cleavage.

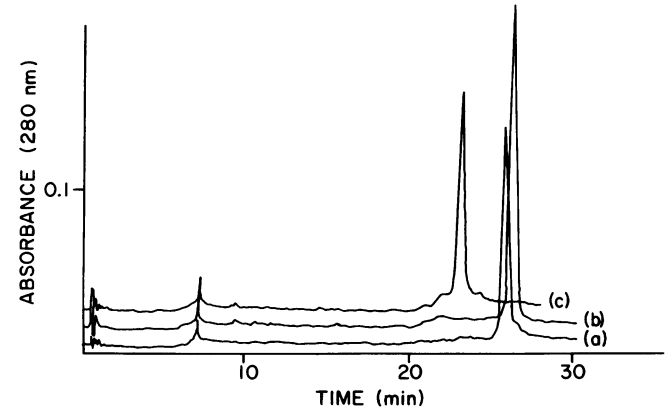

Figure 3. Ion exchange HPLC shows traces of (A) oligonucleotide 1, (B) oligonucleotide 2 and (C) oligoncleotide 3. Oligonucleotide 2 was retained more than oligonucleotide 1, because of, one phosphorothioate diester linkage. Oligonucleotide 3 eluted earlier than oligonucleotide 1 and 2, confirming that in oligonucleotide 3, one of the internucleoside linkages in phosphoramidite, which is non-ionic.

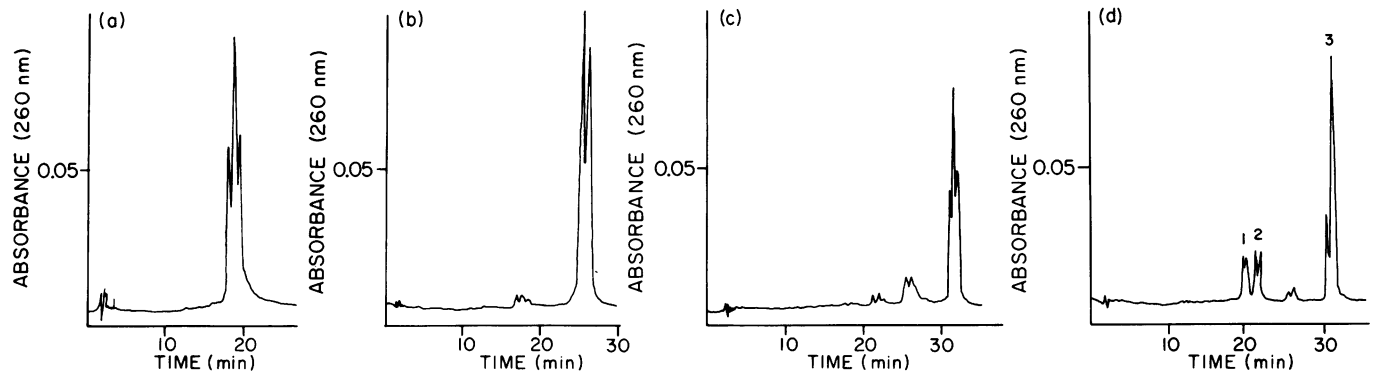

Figure 4. Reversed phase HPLC, traces of (A) ion exchange HPLC-purified oligonucleotide 3 (B) reaction product of oligonucleotide 3 and monobromobimane−oligonucleotide 5, (C) reaction product of oligonucleotide 5 and NHS-ester of biotin, Oligonucleotide 6 and (D) reaction product of oligonucleotide 5 with fluorescein isothiocyanate, Oligonucleotide 7. In chromatogram (D) peak 1 is the hydrolysis product (desulfurized), peak 2 is desulfurized oligonucleotide 5 reacted with fluoroscein isothiocyanate, and peak 3 is oligonucleotide 7 carrying both fluorophores. Peak 3 was also monitored at 385nm and 490nm.

analyzed on an ion exchange HPLC. Oligonucleotide 3 eluted earlier than underivatised oligonucleotide 1 and oligonucleotide 2, confirming that in oligonucleotide 3, one of the internucleoside linkages is phosphoramidate, which is non-ionic (figure 3). The retention time of the peak was not much affected by changing the site of phosphoramidate linkage along the sequence. Reversed phase HPLC analysis of oligonucleotide 3 (ion exchange HPLC purified) gave a multiplet, because of the fact that there are two diasterioisomeric internucleoside linkages (figure 4). Reaction of monobromobimane with oligonucleotide 3 gave a product, oligonucleotide 5, eluting later than oligonucleotide 3. Under the same conditions, oligonucleotide derivatised only with an amine functional at the internucleoside phosphate was unreactive (data not shown). Oligonucleotide 5 was then reacted with the NHS-ester of biotin to yield oligonucleotide 6 in quantitative yield. Oligonucleotide 5 was also reacted with fluoroscein isothiocyanate. Reversed phase HPLC analysis showed disappearance of starting material, and a major peak appeared, eluting later than oligonucleotide 5. This peak was monitored at 260 nm, and also at 380 nm (λmax-bimane), as well as 495 nm (λmax-fluorescein). Oligonucleotide 7, carrying two fluorophores−bimane and fluorescein-exhibited UV-VIS absorbance spectrum and a fluorescence spectrum showing nonradioactive fluorescence resonance energy transfer consistent with the fluorescent labels.

We conclude that attaching two different reporter groups to an oligonucleotide—one specific to thiol and the other specific to an amine can be accomplished at a suitably modified internucleoside phosphate backbone. At the site of introduction of amine specific label, the coupling can be carried out using H-phosphonate chemistry, followed by oxidation with N-trifluoroacetyl alkyldiamine in carbon tetrachloride to generate a phosphoramidate (aminoalkyl) internucleoside linkage. The phosphoramidate internucleoside linkage is stable under oligonucleotide assembly and deprotection conditions. A phosphorothioate diester internucleoside linkage, for attaching thiol specific labels, can be introduced either by oxidizing an H-phosphonate internucleoside linkage with sulfur (as described here in) or by altering the oxidation step with sulfur employing phosphoramidite chemistry (48,49). For introducing a phosphorothioate diester linkage at an internal site, the chemistry of choice is phosphoramidite. In the case of phosphoramidite chemistry, the product of oxidation with sulfur is a phosphorothioate triester, which is stable enough under further coupling steps involved using phosphoramidite chemistry followed by oxidation with iodine to extend the oligomer. [$t_{1/2}$ of desulfurization of phosphorothioate triester in iodine reagent is 24 minutes compared to 7.5 minutes in case of phosphorothioate diester].

This route provides a way for attaching two different labels in an oligonucleotide at one or more specified sites. We have illustrated the method by introducing bimane at a phosphorothioate diester linkage and biotin and fluorescein at a phosphoramidate internucleoside linkage. In other experiments (data not shown) the 1.5-I-AEDANS and 5-iodoacetamido-fluorescein have been attached to the phosphorothioate diester linkage of oligonucleotide 3 and tetramethylrhodamine isothiocyanate to the amine function of oligonucleotide 3 carrying either 1.5-I-AEDANS or fluorescein.

The ability to attach two different reporter groups at specific sites within a DNA sequence would simplify studies involving protein binding, structural analyses and nucleic acid dynamics by nonradiative fluorescence resonance energy transfer (FRET). The energy transfer is dependent on the distance between the two fluorophores (in the size range of 30 to 70Å); the efficiency of transfer (E) is given by $E \alpha [1+(R/Ro)^6]$, where R is the distance between the two fluorophores, and Ro is the distance where the efficiency of transfer is 0.5 (18). Because of its exquisite sensitivity over short distances, nonradiative energy transfer has been called the spectroscopic ruler (18,46). We and others (13−15) have successfully used this method to study DNA-DNA and DNA-RNA interactions. The method described herein for attaching two different fluorophores to an oligonucleotide will allow even small, conformational changes in distances to be quantified using FRET upon interacting with a native binding site *in vitro* as well as *in vivo*.

Bifunctionalization of oligonucleotides, described herein, also provides the means for attaching distinct chemical moities to an oligonucleotide such as an intercalating agent to one functionalized site and chemical agents to modify or cleave target sequences to the other functionalized site in the growing field of 'antisense' oligonucleotides (16,17). These 'third generation' modifications of antisense oligonucleotides offer the possibility of enhancing potency of this new potential type of chemotherapy.

ACKNOWLEDGEMENTS

We thank Dr. D.M. Brown (MRC Laboratory of Molecular Biology, Cambridge U.K.), for critical review of this manuscript, and Dr. Rich Cardullo for assistance in fluorescence measurement. We are also very grateful to Dr. Michael Gait for helpful discussions. This research was supported by a grant from the G. Harold and Leila Y. Mathers Foundation, NCI Cancer Center Core Grant P30 12708-15 and NIAID Cooperative Grant 401 A 124846.

REFERENCES

1. Mathews,J.A. and Kricka,L.J. (1988) Anal. Biochem., 169, 1–25.
2. Landegren,U., Kaiser,R., Caskey,C.T. and Hood,L. (1988) Science, 242, 229–237.
3. Ansorge,W., Sproat,B.S., Stegeman,J., Schwager,C. and Zenke,M. (1987) Nucleic Acid Research, 15, 4593–4602.
4. Brumbaugh,J.A., Middendorf,L.R., Grove,D.L. and Ruth,J.L. (1988) Proc. Natl. Acad. Sci. USA, 85, 5610–5614.
5. Beck,S. and Pohl,F.M. (1984) EMBO J. 3, 2905–2909.
6. Kaiser,R.J., Mackeller,S.L., Vinayak,R.S., Sanders,J.Z., Saavedra,R.A. and Hood,L.E. (1989) Nucleic Acid Research, 17, 6087–6102.
7. Sproat,B.S., Beijer,B. and Rider,P. (1987) Nucleic Acid Research, 15, 6181–6197.
8. Agrawal,S. and Zamecnik,P.C. (Unpublished data.)
9. Loke,S.L., Stein,C.A., Zhang,X.H., Mori,K., Nakanishi,M., Subasingha,C., Cohen,J.S. and Neckers,L.M. (1989) Proc. Natl. Acad . Sci. USA, 86, 3474–3478.
10. Sproat,B.S., Beijer,B., Rider,P. and Neuner,P. (1987) Nucleic Acid Research, 15, 4837–4848.
11. Agrawal,S., (1989) Tet. Lett., 30, 7025–7028.
12. Lamond,A.I., Sproat,B.S., Ryder,U. and Hamm,J. (1989) Cell, 58, 383–390.
13. Cardullo,R.A., Agrawal,S., Flores,C., Zamecnik,P.C. and Wolf,D.E. (1988) Proc. Natl. Acad. Sci. USA, 85, 8790–8794.
14. Agrawal,S., Cardullo,R., Zamecnik,P.C., and Wolf,D.E. (1988) J. Cell Biology, 107, 468.
15. Murchie,A.I.H., Clegg,R.M., Kitzing,E.von., Duckett,D.R., Diekmann,S. and Lilley,D.M.J. (1989) Nature, 341, 763–766.
16. Helene,C. and Toulme,J.J. (1989) In Cohen,J.S. (ed.) Oligonucleotides: Antisense inhibitors of gene expression, Macmillan Press, London, pp. 137–172 and references therein.
17. Knorre, D.G., Vlassov, V.V. and Zarytova, V.F. (1989) In Cohen, J.S. (ed.) oligonucleotides: Antisense inhibitors of gene expression, MacMillan Press, London pp. 173–196 and references therein.
18. Forster,T. (1948) Ann. Phys., 2, 55–75.
19. Chu,B.C.F., Wahl,G.M. and Orgel,L.E. (1983) Nucleic Acid Research, 11, 6513–6529.
20. Kawashima,E.H. and Chollet,A. (1985) Nucleic Acid Research, 13, 1529–1541.
21. Chu,B.C.F. and Orgel,L.E. (1988) Nucleic Acid Research, 16, 3671–3691.
22. Bischoff,R., Coull,J.M. and Regnier,F.E. (1987) Anal. Biochem., 164, 336–344.
23. Ghosh,S.S., Kao,P.M., and Ywoh,D.Y. (1989) Anal. Biochem., 178, 43–51.
24. Zuckermann,R., Corey,D. and Schultz,P. (1987) Nucleic Acid Research, 15, 5305–5321.
25. Jablonski,E., Moomaw,E.W., Tullis,R.H. and Ruth,J.L. (1986) Nucleic Acid Research, 14, 6115–6128.
26. Sproat,B.S., Lamond,A.I., Beijer,B., Neuner,P. and Ryder,U. (1989) Nucleic Acid Research, 17, 3371–3386.
27. Urdea,M.S., Warner,B.D., Running,J.A., Stempien,M., Clyne,J. and Horn,T. (1988) Nucleic Acid Research, 16, 4937–4956.
28. Allen,D.J., Darke,P.L. and Benkovic,S.J. (1989) Biochemistry, 28, 4601–4607.
29. Smith,L.M., Fung,S., Hunkapiller,M.W.S., Hunkapiller,T.J. and Hood,L.E. (1985) Nucleic Acid Research, 13, 2399–2419.
30. Agrawal,S., Christodoulou,C. and Gait,M.J. (1986) Nucleic Acid Research, 14, 6227–6245.
31. Emson,P.C., Arai,H., Agrawal,S., Christodoulou,C. and Gait,M.J. (1989) Meth. Enzymol., 168, 753–761.
32. Arai,H., Emson,P.C., Agrawal,S., Christodoulou,C. and Gait,M.J. (1988) Molecular Brain. Res., 63–69.
33. Connolly,B.A. (1987) Nucleic Acid Research, 15, 3131–3139 and references therein.
34. Nelson,P.S., Gold,R.S. and Leon,R. (1989) Nucleic Acid Research, 17, 7177–7186 and references therein.
35. Haralambidis,J., Duncan,L., Angus,K. and Tregear,G.W. (1990) Nucleic Acid Research, 18, 493–499.
36. Letsinger,R.L., Bach,S. and Eddie,J. (1986) Nucleic Acid Research, 14, 3487.
37. Jager,A., Levy,M.J. and Hecht,S.M. (1988) Biochemistry, 27, 7237–7246.
38. Letsinger,R.L., Singman,C.N., Histand,G. and Salunkhe,M. (1988) J. Am. Chem. Soc., 110, 4470–4471.
39. Agrawal,S., Mayrand,S., Zamecnik,P.C. and Pederson,T. (1990) Proc. Natl. Acad. Sci. USA, 87, 1401–1405.
40. Findanza,J.A. and McLaughlin,L.W. (1989) J. Am. Chem. Soc., 111, 9117–9119.
41. Hodges, R., Conway, N.E. and McLaughlin, L.W. (1989) Biochemistry, 28, 261–267.
42. Agrawal,S. and Tang,J.-Y. (1990) Tet. Lett., 31, 1543–1546.
43. Caruthers,M.H., Barone,A.D., Beaucage,S.L., Dodds,D.R., Fisher,E.F., McBride,L.J., Matteucci,M., Stabinsky,Z. and Tang,J.-Y. (1987) Methods in Enzymology, 154, 287–313.
44. Garegg,P.J., Regberg,T., Stawinski,J. and Stroemberg,R. (1985) Chemica Scripta, 25, 280.
45. Froehler,B., Ng,P. and Matteucci,M. (1986) Nucleic Acid Research, 14, 5399–5407.
46. Stryer,L. (1978) Ann. Rev. Biochem. 47, 819–846.
47. Matayoshi,E.D., Wang,G.T., Krafft,G.A. and Erickson,J. (1989) Science 247, 954–958.
48. Connolly,B.A., Potter,B.V.L., Eckstein,F., Pingoud,A. and Grotjahn,L. (1984) Biochemistry, 23, 3443–3453.
49. Zon,G. (1984) J. Am. Chem. Soc., 106, 6077–6079.