Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1863–1867

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Preparation of Oligonucleotides Without Aldehyde Abasic Sites

Achim H. Krotz,* Hans Gaus, Vasulinga T. Ravikumar and Douglas L. Cole

Isis Pharmaceuticals Inc., 2292 Faraday Avenue, Carlsbad, CA 92008, USA

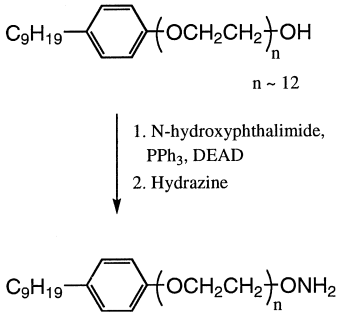
Received 23 March 2001; accepted 30 April 2001

Dedicated to Professor Dr. G. Helmchen on the occasion of his 60th birthday.

Abstract—High-quality oligonucleotides are obtained by selective modification of sequences containing aldehyde apurinic sites with a new chromatographic tag followed by RP-HPLC separation. Hydroxylamine derivative **1** of a water soluble nonionic surfactant modifies oligonucleotides selectively at abasic sites leading to significantly increased retention.

Oligonucleotides and analogues thereof are widely used in molecular biology and diagnostics and as therapeutic agents. Phosphorothioate oligonucleotides in particular have emerged as drugs for treatment of various diseases through antisense mechanisms of action.[1] In an ongoing effort to improve the quality of oligonucleotide drugs, we were interested in eliminating oligonucleotide impurities containing abasic sites. Depurination, formally the cleavage of the *N*-glycosidic bond with concomitant substitution of adenine or guanine by a water molecule, occurs during acid treatment in solid-phase synthesis and, even more importantly, during post-purification removal of the 5′-OH protecting group which is typically 4,4′-dimethoxytrityl.[2] Oligonucleotides containing resultant abasic sites form thermodynamically less stable double and triple helices with complementary oligomers, including target mRNA.[3] Reactions of abasic sites with oligonucleotides, proteins, and intercalators have been reported.[4] Base treatment of abasic oligonucleotides leads to strand scission at the abasic site,[2b,c] however, the shorter fragments formed are difficult to fully remove. Several highly sensitive methods are available to detect apurinic sites,[5] but to our knowledge, there is no efficient purification process reported in the literature for removing oligonucleotide impurities containing abasic sites. In this communication, we report a new reagent that allows detection of oligonucleotides containing aldehyde apurinic sites and quantitative and selective removal from the parent oligonucleotide, using a novel chromatographic tag, thus producing oligonucleotides of increased purity.

A deoxyribose sugar at an abasic site in its ring-closed hemiacetal form (99%) is in equilibrium with its open-chain aldehyde form (1%).[6] Hydroxylamine derivative **1** (Scheme 1) of polyoxyethylene(12)nonylphenyl ether (Igepal™ CO-720, Sigma-Aldrich) is designed to react selectively with aldehyde functionality of oligonucleotide apurinic sites to form a stable oxime derivative[7] (Scheme 2). In the presence of **1**, the equilibrium of ring-closed hemiacetal form and open-chain aldehyde form may be shifted towards the latter so that potentially all 'aldehyde-abasic' sites may react. No strand breaks are expected upon treatment with a hydroxylamine based derivative. The polyoxyethylene chain of **1** renders the surfactant molecule water soluble in order to permit favorable reaction kinetics with oligonucleotides. A chain length of ca. 12 ethylene glycol units provides sufficient hydrophilicity. The aromatic/aliphatic section of the molecule is designed to provide lipophilicity in the modified oligonucleotide to increase retention for



1

Scheme 1. Synthesis of chromatographic tag **1**.

*Corresponding author. Tel.: +1-760-603-3824; fax: +1-760-929-0528; e-mail: akrotz@isisph.com

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00307-9

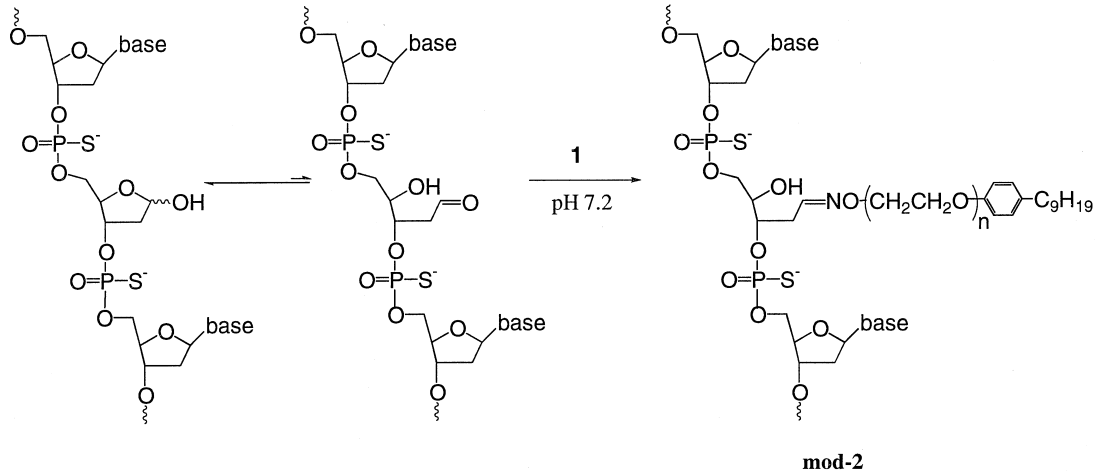
facile separation from unmodified oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC).

Polyoxyethylene(12)nonylphenyl ether is a nonionic surfactant that is commercially available at very low cost. Treatment of polyoxyethylene(12)nonylphenyl ether with *N*-hydroxyphthalimide (1.25 equiv) in the presence of triphenylphosphine (1.25 equiv) and diethyl azodicarboxylate (1.2 equiv) in anhyd THF yielded the *N*-phthalimido derivative in near quantitative yield. Subsequent treatment with hydrazine afforded **1** in good overall yield. MS analysis of polyoxyethylene(12)nonylphenyl ether and **1** showed a molecular weight distribution due to a different number of ethylene glycol units ($n=8$–16) with the maximum at $n=11$. Compound **1** is obtained as a colorless oil that solidifies upon refrigeration.[8]

To illustrate effectiveness of the method described here, we generated apurinic sites in phosphorothioate oligodeoxyribonucleotide PS-d(GCCCAAGCTGGCATCCGTCA) (**2**) by extended treatment with acetic acid.[9] LC–MS spectrum of this mixture (**dep-2**) of parent oligonucleotide and mono- and bis-depurinated oligonucleotides is shown in Figure 1a. The assignment of the masses is given in Table 1. The ES–MS spectrum of **dep-2** shows the parent oligonucleotide **2** at $m/z=2121.4$ $[M-3H]^{3-}$. Two kinds of depurination products are apparent in the spectrum. The most prominent products are caused by displacement of adenine and guanine, respectively, with water leading to 'aldehyde apurinic sites' at $m/z=2082.4$ $[M-3H]^{3-}$ (**2a**) and 2077.0 $[M-3H]^{3-}$ (**2b**), respectively. Due to the extended acid treatment there are also bis-depurinated species detected. Signals at $m/z=2043.6$ (**2e**), 2038.2 (**2f**), and 2032.2 (**2g**) $[M-3H]^{3-}$ are assigned to sequences of **2** in which two adenine, one adenine and one guanine, or two guanine bases are replaced by two water molecules. To a lesser degree, oligonucleotide products formed by elimination of adenine and guanine bases leading to 1,2-didehydroribose products at $m/z=$ 2077.0 (**2c**) and 2071.6 (**2d**), respectively, are detected. The elimination product formed by the loss of adenine overlaps with the substitution product formed by loss of guanine and addition of water.

The chromatographic retention of macromolecules with only minor structural differences, here depurinated sequences and unmodified oligonucleotide is very similar, therefore requiring tailormade purification procedures to achieve separation which allows quantitative recovery of the desired component. Highly selective modification of the chromatographic properties of one component of the mixture through covalent bond formation is the approach described here. Reaction of the *undesired* component with the chromatographic tag is the preferred method as post-purification chemical transformations are avoided.

A solution of **dep-2** (100 mg) in sodium phosphate buffer (2 mL, 0.1 M, pH 7.2) was incubated with a freshly prepared solution of **1** (20 mg) in the same buffer (2 mL) at room temperature.[10] Following reaction of **1** with aldehyde apurinic sites, a stable oxime bond is formed. RP-HPLC analysis of the reaction solution indicated the formation of covalently modified oligonucleotide products (**mod-2**) with longer retention time (t_R 16.5 min) over a period of about 20 h (Fig. 2b) As shown in Figure 2a, the formation of lipophilic species initially increases rapidly then slows down substantially after ca. 10 h indicative of the high selectivity of the reagent for the target aldehydes. The peak area corresponding to **1** decreases rapidly initially and then remains constant. **Mod-2** is chromatographically well separated from the 'purified' oligonucleotide (Fig. 2b) (**pur-2**, t_R 11.5 min) allowing for quantitative separation and recovery of both fractions. LC–MS analysis of isolated **pur-2** shows that the levels of mono- and bis-aldehyde apurinic sites are reduced below the level of detection (Fig. 1b). The small amount of elimination product at m/z 2071 and presumably also at m/z 2076.6, remains unchanged as expected. LC–MS analysis of **mod-2** (Fig. 3) shows two sets of covalently modified oligonucleotides due to the weight distribution of **1**. One set of signals corresponds to oxime derivatives formed by reaction of **1** with deadenylated oligonucleotides **2a** centered at



mod-2

Scheme 2. Equilibrium between ring-closed hemiacetal form and open-chain aldehyde form at an aldehyde apurinic site and selective covalent derivatization of apurinic sequences with chromatographic tag **1**.

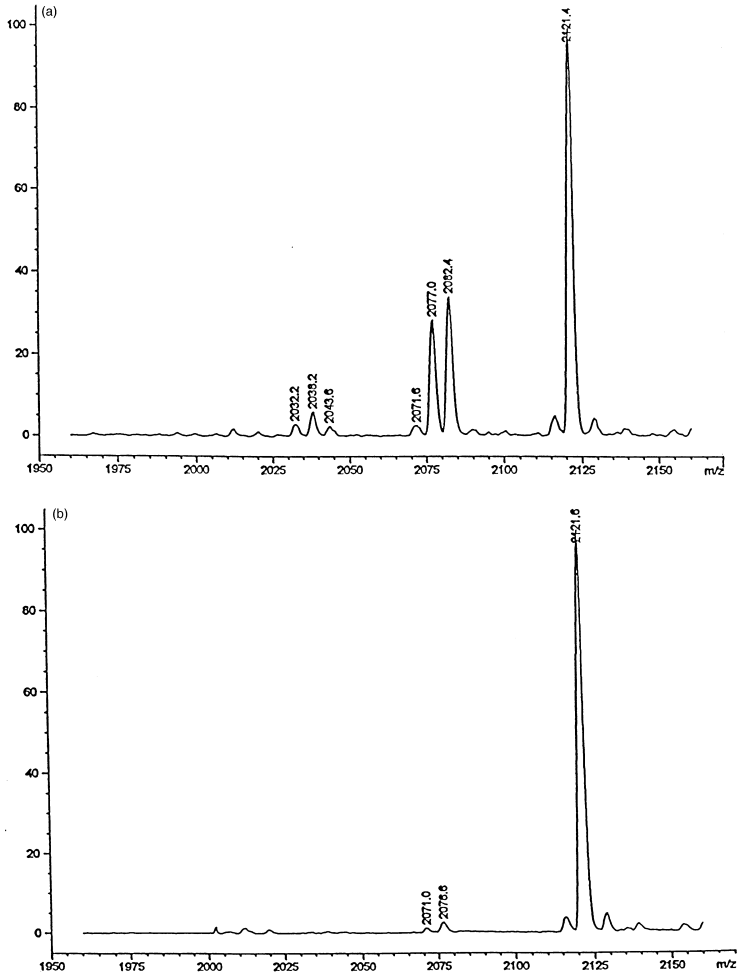


Figure 1. (a) Electrospray ionization mass spectrum of **dep-2**. Shown are the −3 charge states of the parent oligonucleotide **2** (m/z 2121.4), monodepurinated sequences **2a** (m/z 2082.4) and **2b** (m/z 2077.0) and bis-depurinated sequences **2e** (m/z 2043.6), **2f** (m/z 2038.2), and **2g** (m/z 2032.2). m/z 2071.6 is assigned to 1,2-didehydroribose oligonucleotide **2d**. (b) Electrospray ionization mass spectrum of **pur-2**. Note the reduction of levels of aldehyde apurinic sequences **2a**, **2b**, **2e**, **2f**, and **2g** below the level of detection. Signals at m/z 2076.6 and 2071.0 are assigned to 1,2-didehydroribose oligonucleotides **2c** and **2d**.

$m/z = 1737.3$, the other set centered at $m/z = 1744.2$ corresponds to the reaction products of **1** and deguanylated oligonucleotides **2b** (Table 2). The absence of a third set of signals formed by covalent modification of the parent oligonucleotide **2** underscores further the specificity of the method for apurinic sequences.

In summary, the limited resolving power of chromatographic systems for macromolecules of similar composition, in this case oligonucleotides has been addressed by using a novel affinity tag that selectively modifies the chromatographic retention of oligonucleotides containing apurinic sequences. Nonionic surfactant-conjugated hydroxylamine derivative **1** that reacts selectively with open-chain aldehydes in oligonucleotides has been

Table 1. Assignment of molecular masses of **2** and its deletion sequences

	Mass (calcd)		Found
	19H-form	−3 charge state	−3 charge state
PS-d(GCCCAAGCTGGC ATCCGTCA) (**2**)	6368.4	2121.8	2121.4
2−Adenine + water (**2a**)	6251.3	2082.8	2082.4
2−Guanine + water (**2b**)	6235.3	2077.4	2077.0
2−Adenine (**2c**)	6233.3	2076.8	2077.0
2−Guanine (**2d**)	6217.3	2071.4	2071.6
2−2 Adenine + 2 water (**2e**)	6134.1	2043.7	2043.6
2−Adenine−guanine + 2 water (**2f**)	6118.1	2038.4	2038.2
2−2 Guanine + 2 water (**2g**)	6102.1	2033.0	2032.2

Table 2. Assignment of covalently modified deletion sequences

	−4 charge state m/z			
	2a + **1** − water		**2b** + **1** − water	
No. of oxyethylene groups n of **1**	Calcd	Found	Calcd	Found
9	1715.6	1715.3	1711.6	1711.2
10	1726.5	1726.3	1722.5	1722.1
11	1737.6	1737.3	1733.6	1732.6
12	1748.6	1748.2	1744.6	1744.2
13	1759.6	1759.2	1755.6	1754.9
14	1770.6	1770.4	1766.6	1766.1
15	1781.6	1781.5	1777.6	1777.1

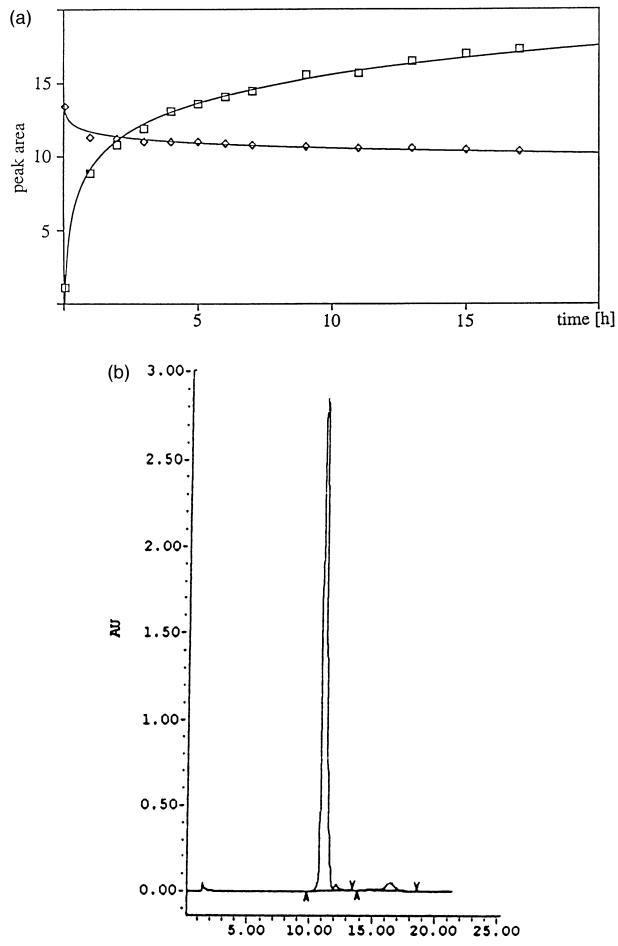


Figure 2. Reaction of **1** and **dep-2** followed by RP-HPLC. (a) Formation of **mod-2** (squares) and disappearance of **1** (diamonds). (b) Chromatographic separation of **pur-2** (t_R 11.5 min) and **mod-2** (t_R 16.5 min). (chromatographic conditions: VYDAC C4 column, gradient 1–99% CH_3CN in NaOAc (0.1 M) in 30 min, flow 1.3 mL/min, det. $\lambda = 254$ nm).

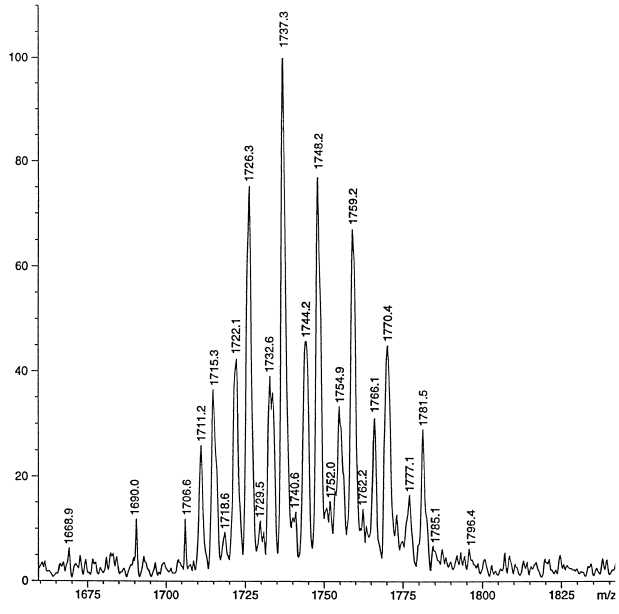


Figure 3. Electrospray ionization mass spectrum of **mod-2**.

described. The hydrophilic 'tail' renders the molecule water soluble, the aromatic/aliphatic 'head' provides lipophilicity to the oligonucleotide conjugate. A stable oxime bond is formed between **1** and the open-chain aldehyde resulting from depurination. The increased lipophilicity of the depurinated oligonucleotide adduct allows for preparative separation and quantitative recovery of oligonucleotides without aldehyde apurinic species. Chromatographic separation offers potential for efficient automated purification. The procedure is characterized by its simplicity and constitutes a convenient method to detect abasic sites in oligonucleotides and separate abasic sequences without leaving shorter fragments behind, both on analytical and on preparative scale.

References and Notes

1. (a) Crooke, S. T. *Antisense Ther. Biotechnol. Genet. Eng. Rev.* **1998**, *15*, 121 (Intercept Ltd. Hampshire, UK). (b) Crooke, S. T. *Antisense & Nucleic Acid Drug Dev.* **1998**, *8*, 115. In August 1998, Isis Pharmaceuticals, Inc., Carlsbad, CA and Ciba-Vision, a division of Novartis AG, Switzerland, receivd FDA approval for Vitravene^{TM} (fomivirsen sodium injectible) for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS.
2. (a) Septak, M. *Nucleic Acids Res.* **1996**, *24*, 3053. (b) Suzuki, T.; Ohsumi, S.; Makino, K. *Nucleic Acids Res.* **1994**, *22*, 4997. (c) Tamm, C.; Shapiro, H. S.; Lipshitz, R.; Chargaff, E. *J. Biol. Chem.* **1953**, *203*, 673.
3. (a) Fukui, K.; Morimoto, M.; Segawa, H.; Tanaka, K.; Shimidzu, T. *Bioconjugate Chem.* **1996**, *7*, 349. (b) Goljer, I.; Withka, J. M.; Kao, J. Y.; Bolton, P. H. *Biochemistry* **1992**, *31*, 11614. (c) Horne, D. A.; Dervan, P. B. *Nucleic Acids Res.* **1991**, *19*, 4963. (d) Bertrand, J. R.; Vasseur, J. J.; Rayner, B.; Imbach, J. L.; Paoletti, J.; Paoletti, C.; Malvy, C. *Nucleic Acids Res.* **1989**, *17*, 10307.
4. (a) Goffin, C.; Brieteux-Gregoire, S.; Verly, W. G. *Biochim. Biophys. Acta* **1984**, *783*, 1. (b) Letellier, R.; Taillandier, E.; Bertrand, J. R.; Malvy, C. *J. Biomol. Struct. Dyn.* **1991**, *9*, 579.
5. (a) Asaeda, A.; Ide, H.; Terato, H.; Takamori, Y.; Kubo, K. *Anal. Chim. Acta* **1998**, *365*. (b) Makrigiorgos, G. M.; Chakrabarti, S.; Mahmood, A. *Int. J. Radiat. Biol.* **1998**, *74*, 99. (c) Nakamura, J.; Walker, V. E.; Upton, P. B.; Chiang, S.-Y.; Kow, Y. W.; Swenberg, J. A. *Cancer Res.* **1998**, *58*, 222. (d) Chen, B. X.; Kubo, K.; Ide, H.; Erlanger, B. F.; Wallace, S. S.; Kow, Y. W. *Mutation Res.* **1992**, *273*, 253. (e) Talpaert-Borle, M.; Liuzzi, M. *Biochim. Biophys. Acta* **1983**, *740*, 410.
6. Wilde, J. A.; Bolton, P. H.; Mazumder, A.; Manoharan, M.; Gerlt, J. A. *J. Am. Chem. Soc.* **1989**, *111*, 1894.
7. Coombs, M. M.; Livingston, D. C. *Biochim. Biophys. Acta* **1969**, *174*, 161.
8. **1**: A solution of Igepal^{TM} CO-720 (30.0 g, 40 mmol) in anhyd THF (80 mL, dried with mol sieves 3 Å) is added to a solution of triphenyl phosphine (13.3 g, 50 mmol) and *N*-hydroxyphthalimide (8.2 g, 50 mmol) in anhyd THF (250 mL). Diethyl azodicarboxylate (8.4 g, 48 mmol) is added dropwise over a period of 15 min (exothermic reaction). After 14 h, the solution is concentrated in vacuo, the oily residue is dissolved in dichloromethane (500 mL) and extracted with water (200 mL). The organic layer is dried over Na_2SO_4. The solvent is evaporated and the remaining oil is dissolved in ethyl acetate/hexanes (100 mL, 8:2, v/v) and purified by flash chromatography (silica, 270 g, eluents: ethyl acetate/hexanes (8:2, 1 L), ethyl acetate (1.5 L), ethyl acetate/methanol (1.5 L, 9:1). Product

fractions are combined and the solvent is removed in vacuo to afford the *N*-phthalimido derivative of Igepal™ CO-720 as colorless oil in near quantitative yield. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.4–1.8 (19H, aliph.), 3.4–4.4 (48H, OCH_2), 6.7–7.2 (4H, aromat.), 7.6–7.9 (4H, phthalimido) ppm. The *N*-phthalimido derivative of Igepal™ CO-720 (13.5 g) was dissolved in THF (60 mL), cooled to 0 °C and anhyd hydrazine (1 mL) was added dropwise over a period of 10 min. The solution was then stirred for 1 h at rt. Diethyl ether (150 mL) was added, and the mixture was kept at −20 °C overnight. The mixture was filtered and the liquid phase was evaporated. The residue was purified by silica gel column chromatography (column dimensions 10×5 cm, ethyl acetate/methanol 98:2 to 90:10) to give 9.6 g **1** as colorless oil, which solidfies upon cooling. HPLC (Phenomenex Luna C18, 3 μ, 150×4.6 mm, gradient: water/acetonitrile 60:40 to 0:100 in 15 min, 260 nm): t_R 17.9 min. MS: ($n=8$, $C_{31}H_{58}O_9N$, M + H) calcd 588.8, found 588.5, ($n=9$, $C_{33}H_{62}O_{10}N$, M + H) calcd 632.9, found 632.4, ($n=10$, $C_{35}H_{66}O_{11}N$, M + H) calcd 676.9, found 676.5, ($n=11$, $C_{37}H_{70}O_{12}N$, M + H) calcd 721.0, found 720.5, ($n=12$, $C_{39}H_{74}O_{13}N$, M + H) calcd 765.0, found 764.5, ($n=13$, $C_{41}H_{78}O_{14}N$, M + H) calcd 809.1, found 808.5, ($n=14$, $C_{43}H_{82}O_{15}N$, M + H) calcd 853.1, found 852.7, ($n=15$, $C_{45}H_{86}O_{16}N$, M + H) calcd 897.2, found 896.7, ($n=16$, $C_{47}H_{90}O_{17}N$, M + H) calcd 941.2, found 940.7.

9. The method described here works equally well with 'untreated' oligonucleotides.

10. Phosphorothioate oligodeoxyribonucleotide **dep-2** (100 mg) in Na-phosphate buffer (pH 7.2, 0.1 M, 2 mL) and **1** (20 mg) in Na-phosphate buffer (2 mL) were mixed and kept at rt for 20 h. Ethanol (40 mL) was added and the mixture was kept at −20 °C overnight. The precipitated oligonucleotide was isolated by centrifugation and purified by RP-HPLC (Waters Bondapak C18, 100×25 mm). A portion of the oligoncleotide (820 OD_{260}) was dissolved in water and was loaded at 5% CH_3CN/95% triethylammonium acetate (0.1 M) on the column. **Pur-2** was eluted at 20% CH_3CN and **mod-2** was eluted at 75% CH_3CN. Evaporation, followed by ethanol precipitation gave **pur-2** (628 OD_{260}) and **mod-2** (130 OD_{260}).