Immunospecific Reduction of Antioligonucleotide Antibody-Forming Cells with a Tetrakis-oligonucleotide Conjugate (LJP 394), a Therapeutic Candidate for the Treatment of Lupus Nephritis

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Received December 27, 1994[⊗]

A discrete tetravalent conjugate, **7a** (LJP 394), consisting of four oligonucleotides attached to a common carrier or platform was prepared. Single-stranded oligonucleotide 20-mers consisting of alternating deoxycytidine-deoxyadenosine nucleotides, $(CA)_{10}$, were attached to a tetrabromoacetylated platform by displacement with sulfhydryl-terminated linkers. The tetrabromoacetylated platform **3a** was synthesized in three steps using triethylene glycol bis(chloroformate). The single-stranded conjugate was characterized by polyacrylamide gel electrophoresis, DNA sequencing, phosphate analysis, carbon and nitrogen combustion analysis, and correlation of stoichiometry to conversion in the conjugation process. HPLC and capillary electrophoretic methods were developed to evaluate purity. The tetrakis, single-stranded conjugate was annealed with a stoichiometric amount of a complementary single-stranded oligonucleotide 20-mer consisting of alternating thymidine-deoxyguanosine nucleotides, $(TG)_{10}$. The double-stranded conjugate LJP 394 was characterized by melt temperature and hyperchromicity, phosphate analysis, and carbon and nitrogen combustion analysis. LJP 394 inhibits binding of DNA to anti-double-stranded oligonucleotide antibodies and reduces anti-oligonucleotide-specific plaque (antibody)-forming cells in an immunized mouse model by a proposed mechanism involving cross-linking B cell surface immunoglobins.

Introduction

Multiple copies of a hapten attached covalently to a nonimmunogenic polymeric backbone can down regulate, or tolerize, those B lymphocytes producing antibodies that bind the hapten, rendering them nonresponsive to haptenated immunogens.[1] The result is a decrease in the amount of antibody produced against the hapten. Molecules such as these, which suppress antibody production by B cells, are called toleragens. For example, 2,4-dinitrophenyl (DNP) groups attached to the D-lysines in polymers consisting of D-amino acids can suppress IgG production in animals which have been immunized with DNP-derivatized proteins. Such findings suggested a therapeutic approach for treatment of autoimmune disorders by specifically tolerizing B cells. Systemic lupus erythematosus (SLE) is characterized by antibodies to a number of nuclear antigens. Specifically, antibodies to double-stranded DNA (anti-ds-DNA) are thought to cause lupus nephritis.[2] We have shown that synthetic double-stranded oligonucleotides (ds-ON) cross-react with anti-ds-DNA.[3] Our approach toward a therapy for SLE involves the use of ds-ON conjugates with nonimmunogenic carriers, also referred to as platforms, to inactivate B cells which synthesize anti-ds-DNA antibodies.

We recently reported the synthesis of a tetrakis conjugate, LJP 249, which consists of four double-stranded oligonucleotides attached to a poly(ethylene glycol) "valency" platform.[4] That conjugate was used to demonstrate tolerance in an immunized mouse model. We have since prepared a variety of analogues on various polymeric and nonpolymeric platforms with the premise that use of a smaller platform would yield fully defined conjugates, which would facilitate manufacturing, analysis, purity, and, ultimately, pharmaceutical regulatory issues.

We now wish to report on the synthesis and characterization of a new conjugate, **7a** (LJP 394), which has entered human clinical trials. This conjugate consists of four double-stranded oligonucleotides attached to a new fully defined, tetrakis platform consisting of triethylene glycol which has been modified with alkylamide-branching groups used as tethers for attachement of oligonucleotides.

Discussion

In our continuing efforts to develop a therapy for lupus nephritis, our objective has been to develop a conjugate of four double-stranded oligonucleotides on a completely defined, nonpolymeric platform. LJP 394 (**7a**) is one such analogue, which was selected for its ability to tolerize anti-ds-DNA antibody-forming cells. In addition to its biological activity, it was selected as our primary therapeutic candidate on the basis of the following criteria. (a) The platform, with four thiol-reactive groups attached, can be synthesized efficiently and in high purity. (b) The platform has sufficient solubility in water to undergo conjugation reactions. (c) The conjugate and its expected metabolites are predicted to be nontoxic.

The rational for the design of LJP 394 was based on our previous work involving LJP 249, which has a poly(ethylene glycol)-based platform.[4] Our objective was to replace the polymer-based platform with a discrete nonpolymeric platform. The oligonucleotide length and sequence, duplexes of 20 base pairs (10 repeating CA

[⊗] Abstract published in *Advance ACS Abstracts*, May 1, 1995.

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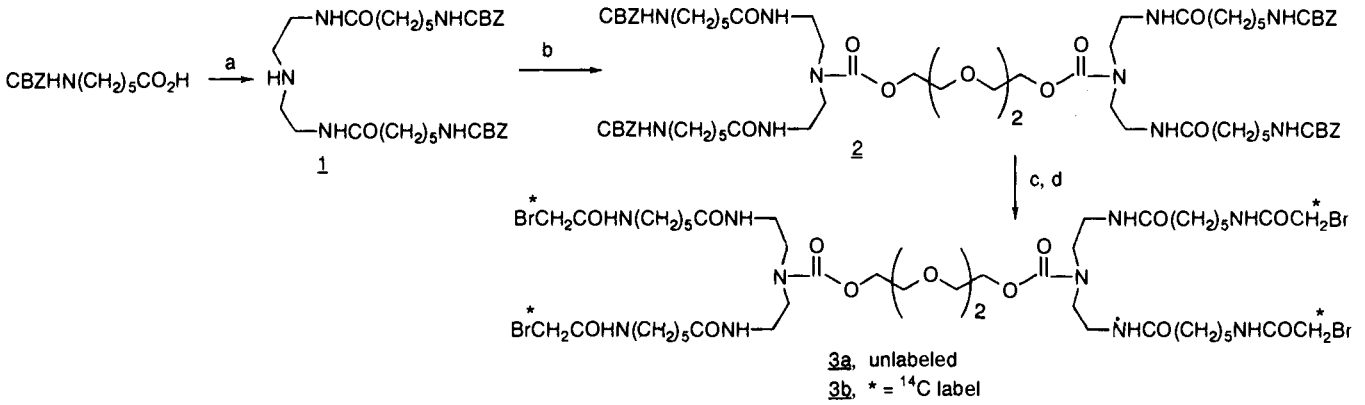
Scheme 1. Synthesis of Bromoacetylated Platform[a]



[a] (a) Carbonyldiimidazole, diethylenetriamine, EtOAc; (b) triethylene glycol bis(chloroformate), pyridine, DMF; (c) Pd/C, cyclohexene, EtOAc; (d) 4-nitrophenyl bromoacetate, $NaHCO_3$, dioxane, H_2O.

units base paired with 10 repeating TG units),[5] was not changed. This sequence was chosen for LJP 249 on the basis of the ability of unconjugated duplexes to bind human SLE serum.[3,6] The rational for choosing to attach four copies of the oligonucleotide to the nonimmunogenic carrier was also a carryover from LJP 249 and was based on the strategy of cross-linking surface antibodies on B cells. While an oligovalent conjugate was desired, we wanted to limit the size of the conjugate in order to avoid producing a T-independent antigen.[7] In addition, the valency was limited by the need to synthesize a discrete, isolatable compound. Since there are possible side reactions in the conjugation step, the more reactive attachment points there are, the more difficult it becomes to achieve a reasonable yield of fully substituted and fully characterizable material. By branching each end of a bifunctional molecule (triethylene glycol), we were able to attach four copies of oligonucleotides.

The synthesis of the bromoacetylated platform, compound *3*, is diagramed in Scheme 1. CBZ-aminocaproic acid is commercially available. Activation of 2 equiv of CBZ-aminocaproic acid with carbonyldiimidazole followed by addition of 1 equiv of diethylenetriamine in the presence of 2 equiv of Et_3N resulted in the selective formation of **1**, which was isolated by crystallization in 75% yield. Triethylene glycol bis(chloroformate) was added to 2 equiv of compound 1 in the presence of pyridine in DMF to provide compound **2**. The CBZ protecting groups were removed using catalytic transfer hydrogenolysis[8] with palladium on carbon in ethanol and cyclohexane, and the resulting tetraamino platform was acylated with 4-nitrophenyl bromoacetate to provide **3a**. 4-Nitrophenyl bromoacetate was prepared by condensing bromoacetic acid with 4-nitrophenol in the presence of DCC.[9] The ^{14}C-labeled platform **3b** was prepared similarly from [^{14}C]bromoacetic acid.[10]

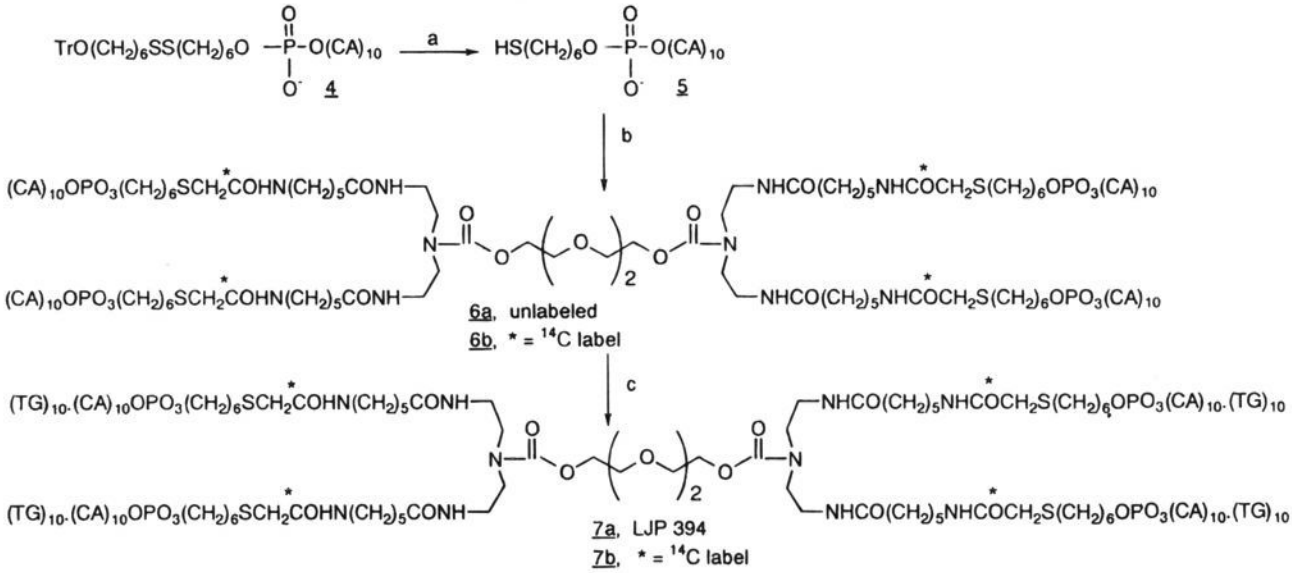
Compound **4**, an oligonucleotide 20-mer comprised of the repeating sequence of deoxycytidine–deoxyadenosine units, $(CA)_{10}$[5], with a disulfide-protected thiol linker attached to the 5′-end, was prepared as previously described.[4] There are numerous examples of oligonucleotides modified with thiol linkers that have been attached to other molecules to form oligonucleotide conjugates.[11] Our approach allows us to use the trityl group of **4** as a hydrophobic handle which facilitates purification of modified full length oligonucleotide from failure sequences which do not contain the 5′-linker. A thiol is subsequently generated by reduction of the disulfide bond under mild conditions and in high yield.

Scheme 2 describes the conjugation and annealing processes used to prepare LJP 394. Reduction of disulfide **4** was accomplished with tributylphosphine[12] in pH 5 sodium acetate buffer, giving rise to thiol-modified oligonucleotide **5**. Compound **5** was isolated by precipitation using an equal volume of isopropyl alcohol.[13]

A series of small scale conjugation reactions were monitored by HPLC[14] to determine optimal conditions and study the effect of stoichiometry on product distribution. Optimal conditions for the conjugation reaction were determined to be addition of a solution of 1 equiv of platform **3a** in 9/1 methanol/H_2O to 4 equiv of **5** in 0.1 M pH 10 sodium borate which had been sparged with helium to remove oxygen to give **6a**. The reaction required a pH above 8 to proceed at a reasonable rate. At pH 10, the reaction was complete in 1–2 h as evidenced by no further conversion to product. Prolonged reaction led to no degradation of product or change in HPLC profile, so we now generally allow the reaction to go overnight to insure completeness.

The single-stranded conjugate **6a** was characterized by correlation of stoichiometry to conversion, polyacrylamide gel electrophoresis, DNA sequencing, phosphorus analysis, carbon and nitrogen combustion analysis, and mass spectrometry. The fully substituted conjugate, with 4 oligonucleotides/platform, was initially identified by the HPLC profile of reactions substoichiometric in oligonucleotide.[15] Addition of substoichiometric amounts of **5** (2.16–3.88 equiv) leads to mixtures containing varying amounts of conjugates with fewer than four oligonucleotides attached (see Figure 1). As the ratio of **5** to **3a** became closer to 4 to 1 (stoichiometric), the peaks of lesser substitution or shorter retention time were converted to the longest retention time peak. When more than 4 equiv were added, there was no evidence of further conversion to higher molecular weight species. These results indicated that the product of retention time of 14.6 min was the desired fully substituted tetramer. Polyacrylamide gel electrophoresis supported the structure **6a**, in that the product ran more slowly than the reference 20-mers, $(TG)_{10}$ and compound **4**, as expected for a molecule of that size (see Figure 2). The base sequence was confirmed by Maxam–Gilbert sequencing.[16] Further support for the structure **6a** was obtained by electrospray mass spectrometry.[17]

Scheme 2. Synthesis of Oligonucleotide Conjugate[a]



[a] (a) Tributylphosphine, pH 5 100 mM sodium acetate; (b) **3**, pH 10 100 mM sodium borate; (c) $(TG)_{10}$, pH 7.2 phosphate-buffered saline.

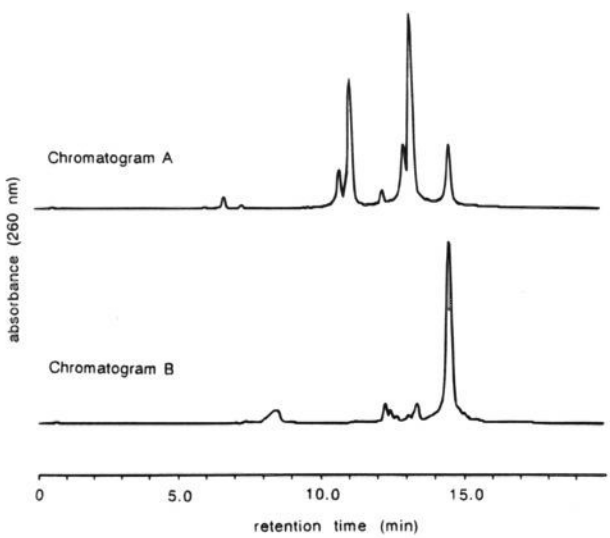


Figure 1. HPLC chromatograms of crude conjugate **6a**: chromatogram A, 2.16 mol equiv of **5**/mol of **3a**; chromatogram B, 3.88 mol equiv of **5**/mol of **3a**; reaction profiles at 1 h time point; GenPak FAX column (60 °C, 1 mL/min, 260 nm; gradient, (A) 0.05 M pH 7.5 sodium phosphate, 1/9 MeOH/H_2O, (B) 0.05 M pH 7.5 sodium phosphate, 1 M NaCl, 1/9 MeOH/H_2O; 30–70% B over 0–16 min).

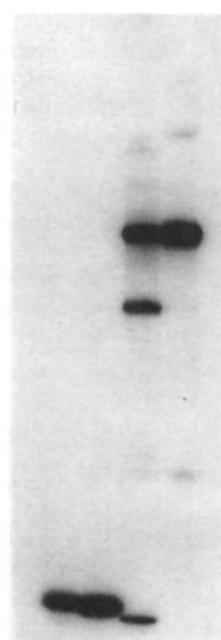


Figure 2. Autoradiogram of an 8% polyacrylamide gel, 3′-end labeled with ^{32}P and terminal transferase: lane A, $(TG)_{10}$; lane B, compound **4**; lane C, crude reaction; lane D, purified **6a**.

The extinction coefficient for **6a** was determined by three independent methods:[18] correlation of absorbance at 260 nm to (a) the phosphorus analysis,[19] (b) the carbon and nitrogen combustion analyses, and (c) the ^{14}C counts in a preparation of **6b**, which was made using 4-nitrophenyl bromo[1-^{14}C]acetate[10] of known specific activity. All three calculated extinction coefficients agreed to within 3%.

LJP 394 (**7a**) was prepared by annealing single-stranded conjugate **6a** with a slight excess of complementary $(TG)_{10}$. The two components were mixed in PBS, and the solution was heated to 70 °C and allowed to cool slowly to room temperature. The mixture was analyzed by HPLC to ensure that there was a slight excess of $(TG)_{10}$. If no excess $(TG)_{10}$ was detected, the process was repeated after adding more $(TG)_{10}$.

The extinction coefficient for **7a** (LJP 394) was determined by three methods:[18] correlation of absorbance at 260 nm to (a) the phosphorus analysis,[19] (b) the carbon combustion analysis, and (c) the ^{14}C counts in a preparation of **7b**, which was made by annealing **6b** with $(TG)_{10}$. All three calculated extinction coefficients agreed to within 4%.

Purities of both the single-stranded conjugate **6a** and the double-stranded conjugate **7a** (LJP 394) were examined by a number of methods. The methods which best resolve **6a** are ion exchange HPLC and capillary electrophoresis (CE) using polyacrylamide gel-filled capillaries. Ion exchange HPLC has proved to be the method of choice for analyses of both single- and double-

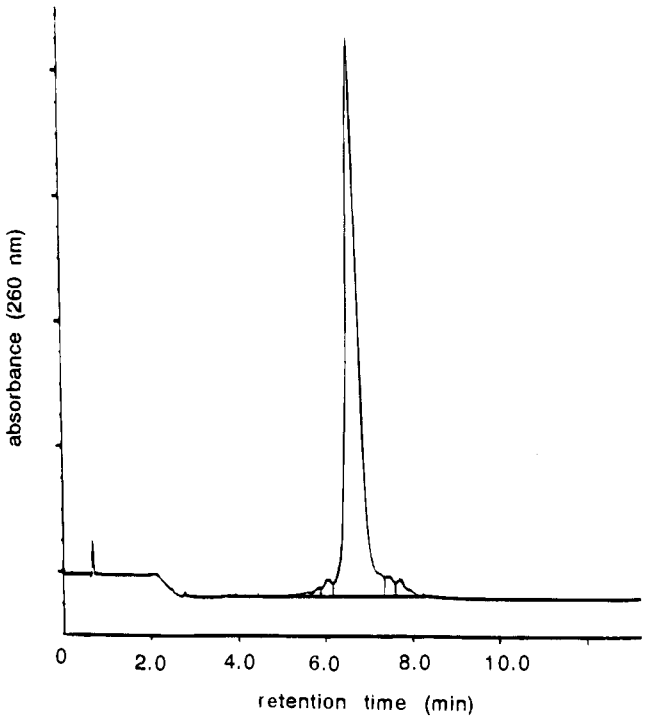


Figure 3. HPLC chromatogram of purified single-stranded conjugate **6a** on a NucleoPac column (55 °C, 2.5 mL/min, 260 nm; gradient, (A) pH 7.5 50 mM sodium phosphate, 1 mM EDTA, 5/95 CH_3CN/H_2O, (B) pH 7.5 50 mM sodium phosphate, 1 mM EDTA, 1.0 M NaCl, 5/95 CH_3CN/H_2O; 30% B, 0–1 min; 30–71% B, 1–1.5 min; 71–80% B, 1.5–12 min). Shoulders on front and back of peak are thought to be related species missing one or more bases on one or more of the oligonucleotides.

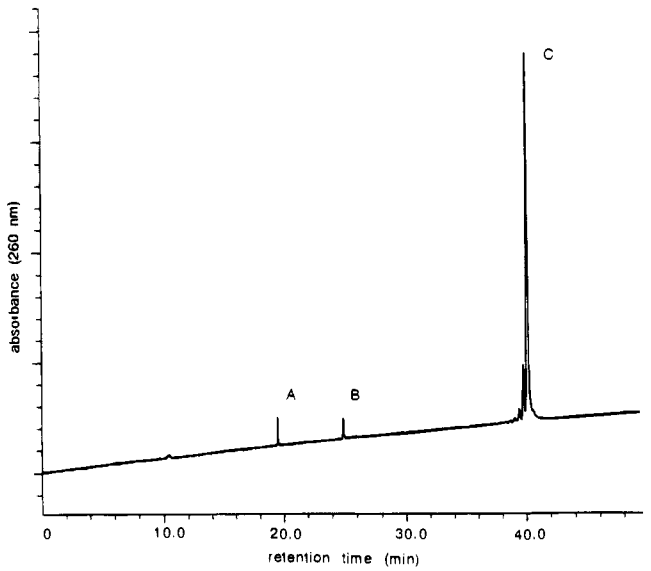


Figure 4. Capillary electrophoretogram of purified **6a** on a Beckman P/ACE 2100 instrument using a rigid polyacrylamide-filled capillary (100 μm × 37 cm; Beckman Instruments; PN 338480, 30 °C, 260 nm; field strength, 300 V/cm; electrokinetic injection, 6 s at 7.5 kV) with oligonucleotide standards T_{20} and T_{40}: A, T_{20}; B, T_{40}; C, **6a**.

stranded conjugates. We found it difficult to maintain reproducibility using the CE method. Figure 3 shows an ion exchange HPLC chromatogram of **6a**, Figure 4 shows a typical CE chromatogram of **6a**, and Figure 5 shows an ion exchange HPLC chromatogram of **7a** (LJP 394).

The serum stability of LJP 394 was examined using agarose gels, visualized with ethidium bromide. LJP

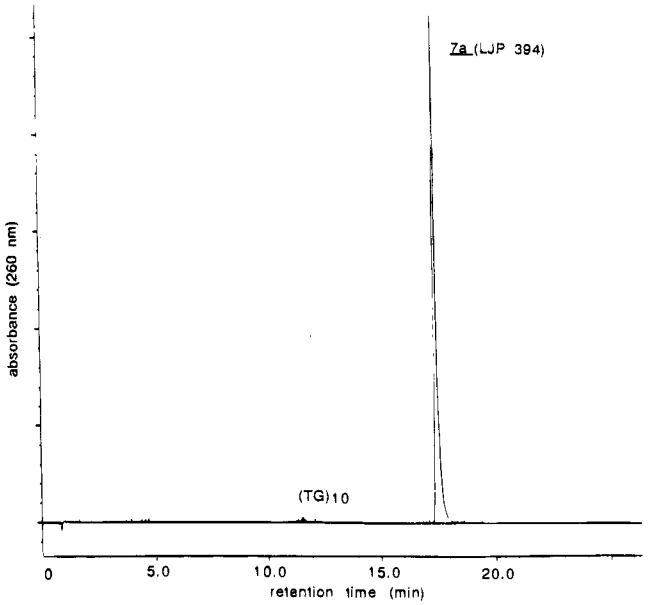


Figure 5. HPLC chromatogram of double-stranded conjugate **7a** (LJP 394) on a GenPak FAX column (40 °C, 0.75 mL/min, 260 nm; gradient (A) pH 7.5 50 mM sodium phosphate, 1 mM EDTA, 1/9 $MeOH/H_2O$, (B) pH 7.5 50 mM sodium phosphate, 1 mM EDTA, 1.0 M NaCl, 1/9 $MeOH/H_2O$; 20–82% B, 0–22.11 min).

394 was found to be relatively stable against human serum nucleases in that the mobility or intensity of the bands did not change after 4 h of incubation at 37 °C in serum from normal or lupus donors. The compound was completely degraded, however, within 2–4 h by sera from lupus mice. No mammalian cellular nucleases were studied, since the molecule is thought to operate on the exterior (surface antibody receptors) of B cells and does not have to enter cells for its biological activity.

***In Vivo* Biology.** Mice (C57BL/6) were immunized (as previously described)[4] with a conjugate of double-stranded oligonucleotides $(CA)_{25}$·$(TG)_{25}$ attached to KLH. Neither DNA nor synthetic oligonucleotides are immunogenic because they lack T cell epitopes and cannot generate the necessary help from CD4[+] T cells required for a functional antibody response. The purpose of making a conjugate between the highly immunogenic protein KLH and synthetic ds-ON was to provide the ds-ON with T cell epitopes. Since the B cell repertoire of C57BL/6 mice recognizes the ds-ON, it can mount an antibody response against the ds-ON-KLH conjugate. These antibodies are of high enough affinity to give a positive Farr assay where the radioactive antigen is present at 10^{-8} M.

Three weeks after immunization, the mice were injected with LJP 394. A control group was injected with saline after the immunization. Five days later, mice in both groups were boosted with the oligonucleotide–KLH conjugate. Four days later, the spleens were harvested and the number of anti-ds-ON antibody-forming cells was determined. The results, shown in Table 1, show that the treatment of the immunized mice with LJP 394 significantly reduced the number of anti-ds-ON antibody-forming cells in a dose-dependent manner. Thus, these mice were rendered unresponsive (tolerant) to further challenge with an immunogenic form of the oligonucleotide.

The specificity of the treatment was demonstrated with measurements of anti-ds-ON antibodies and anti-KLH antibodies in serum from ds-ON-KLH immunized

Table 1. Reduction in the Number of Double-Stranded Oligonucleotide-Specific, IgG, Antibody-Forming Cells in Mice Treated with Compound **7** (LJP 394)

	antibody-forming cells/10^6 spleen cells[a]					
	experiment 1[c]			experiment 2[c]		
dose[b] (nmol)	mean	(SD)	reduction[d] (%)	mean	(SD)	reduction[d] (%)
none	5562	(2570)		5899	(344)	
5.058	982	(1871)	82.3*	3413	(1604)	42.1
1.686	1867	(1335)	66.4*	222	(725)	96.2*
0.562	2247	(1606)	59.6*	1492	(2264)	74.7*
0.187	6109	(2545)	0	5421	(832)	8.1
0.062	4045	(1411)	27.3	5077	(1946)	13.9
0.021	4578	(2475)	17.7	7023	(679)	0
0.007	5930	(847)	0	4159	(2688)	29.5

[a] The number of ds-ON-specific antibody-forming cells was determined in two separate experiments. [b] Dose of LJP 394 per mouse (MW 54 172 g/mol). [c] The data were analyzed by analysis of variance (ANOVA). The groups that were significantly different from the control group are marked with an asterisk. For experiment 1, $P = 0.0117$, and for experiment 2, $P = 0.0001$. [d] Percent reduction was determined by dividing the difference between the number of antibody-forming cells in the treated group and the control group by the number of antibody-forming cells in the control group and multiplying by 100.

mice which had been tolerized with LJP 394. There was no reduction in the anti-KLH (carrier) response as determined in an ELISA assay;[20] however, there was a significant dose-dependent reduction in the anti-ds-ON response as determined by a Farr assay[21] with a mean ED_{50} value of 21 ± 14 ug/mouse (0.389 ± 0.259 nmol/mouse). Thus, the toleragen was specific for down regulating the anti-ds-ON antibody response without affecting the immune response to the protein carrier in the immunogen KLH.

In conclusion, we have synthesized and characterized a nonpolymeric, oligovalent double-stranded oligonucleotide–platform conjugate, **7a** (LJP 394). This conjugate retains the biological activity of its polymeric, poly(ethylene glycol)-based prototype, in that it reduces anti-double-stranded oligonucleotide antibody-forming spleen cells when injected ip in mice which have been immunized to make anti-double-stranded oligonucleotide antibodies. These, and other, results have led us to initiate clinical trials as a therapy for lupus nephritis.

Experimental Section

N-CBZ-aminocaproic acid, carbonyldiimidazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI), dicyclohexylcarbodiimide (DCC), diethylenetriamine, triethylene glycol bis(chloroformate), bromoacetic acid, and 4-nitrophenol were purchased from Aldrich Chemical Co.

Silica gel (230–400 mesh ASTM) was purchased from Baxter. Q-Sepharose was purchased from Pharmacia. Fractogel EMD-DEAE 650(S) (particle size 0.025–0.040 mm) was purchased from EM Separations. ProteinPac Q-HR15 ion exchange packing was purchased from Waters. GenPak FAX HPLC columns were purchased from Waters, and NucleoPac PA-100 HPLC columns (P/N 43010) were purchased from Dionex. Shodex HPLC columns KW802.5 were purchased from Shoko Co. Ltd. TLC was performed on silica gel TLC plates (5554) manufactured by EM Separations. Phosphate-buffered saline (PBS), pH 7.2, was prepared by dissolving 175 g of NaCl, 6.5 g of $NaH_2PO_4{\cdot}H_2O$, and 40.9 g of $Na_2HPO_4{\cdot}7H_2O$ in H_2O and diluting to a final volume of 20 L.

Melting points are reported uncorrected. NMR spectra were recorded on a Bruker AC-300 spectrometer with broad-band probe. DNA synthesis was performed on a Milligen 8800 prep scale DNA synthesizer following the manufacturer's protocols for DNA phosphoramidite synthesis. UV absorbances were measured on a Perkin-Elmer λ-4 spectrophotometer. Melt curves and hyperchromicity were determined on a Varian Cary 3E spectrophotometer. Elemental analysis were performed by Desert Analytics of Tucson, AZ. Mass spectra were obtained from the Mass Spectroscopy Lab, Department of Chemistry, University of California, Berkeley, CA. Sequencing of oligonucleotides was performed by a modification of the Maxam–Gilbert method.[16,22]

Bis(phenylmethyl) 8,16-Dioxo-2,9,12,15,22-pentaazatricosanedioate, 1. A solution of 5.05 g (19.0 mmol) of *N*-(carbobenzyloxy)-6-aminohexanoic acid and 3.09 g (19.0 mmol) of carbonyldiimidazole in 25 mL of EtOAc was stirred for 1.5 h at room temperature, and 1.02 mL (982 mg, 9.52 mmol) of diethylenetriamine was added followed by 2.65 mL (1.93 g, 19.0 mmol) of Et_3N. The mixture was stirred for 4 h, and the solid product which precipitated was collected by filtration. Recrystallization (MeOH/EtOAc) gave 4.27 g (75%) of 1 as a fine grainy solid: mp 132–133 °C; ^{1}H NMR ($CDCl_3$) δ 1.33 (m, 4H), 1.52 (m, 4H), 1.64 (m, 4H), 2.18 (t, 4H), 2.73 (t, 4H), 3.16 (m, 4H), 3.35 (m, 4H), 4.96 (bd, s, 2H), 5.09 (s, 4H), 6.13 (bd s, 2H), 7.33 (s, 10H); ^{13}C NMR (CD_3OD) δ 26.4, 27.3, 30.5, 36.9, 39.8, 41.5, 48.5, 67.2, 128.6, 128.8, 129.3, 138.3, 158.7, 176.2; IR (KBr pellet, cm^{-1}) 1265, 1533, 1629, 1686, 2946, 3316, 3346; MS (FAB, NBA matrix) *m/z* 598 (MH^+) 490, 320; UV (MeOH) 206 (ε = 1365), 254 (ε = 244), 258 (ε = 310), 264 (ε = 255). Anal. ($C_{32}H_{47}N_5O_6$) C, H, N.

Bis(phenylmethyl) 8,13,24,29-tetraoxo-12,25-bis[2-[[1-oxo-6-[[(phenylmethoxy)carbonyl]amino]hexyl]amino]-ethyl]-14,17,20,23-tetraoxa-2,9,12,25,28,35-hexaazahexatriacontanedioate, 2. A mixture of 2.99 g (5.0 mmol) of 1 and 412 μL of pyridine in 15.4 mL of DMF was heated until a clear solution was obtained and then kept at 40 °C. To the mixture was added dropwise over 1 min 515 μL (690 mg, 2.5 mmol) of triethylene glycol bis(chloroformate). The mixture immediately formed a precipitate. The mixture was stirred for 1 h at 40 °C and partitioned between 30 mL of CH_2Cl_2 and 20 mL of water. The CH_2Cl_2 layer was washed with 20 mL of water, dried (Na_2SO_4), filtered, and concentrated. The resulting residue was purified by silica gel chromatography (gradient, 3% MeOH/CH_2Cl_2 to 15% MeOH/CH_2Cl_2) to provide an oil which was crystallized from CH_2Cl_2/EtOAc/heptane to give 2.10 g (60%) of **2** as a white solid: mp 83–85 °C; TLC R_f 0.15 (89/10/1 CH_2Cl_2/MeOH/HOAc); ^{1}H NMR ($CDCl_3$) δ 1.31 (m, 8H), 1.52 (m, 8H), 1.62 (m, 8H), 2.20 (m, 8H), 3.20 (m, 8H), 3.39 (s, 16H), 3.62 (s, 4H), 3.68 (m, 4H), 4.26 (m, 4H), 5.08 (s, 8H), 5.32 (bd s, 4H), 7.31 (bd s, 4H), 7.37 (s, 20H); ^{13}C NMR ($CDCl_3$) δ 25.1, 26.2, 26.4, 29.6, 36.0, 36.2, 38.5, 38.8, 40.8, 64.5, 66.4, 69.1, 70.3, 128.0, 128.4, 136.7, 156.5, 156.9, 173.6; IR (KBr pellet, cm^{-1}) 1140, 1238, 1268, 1548, 1642, 1687, 2934, 3035, 3065, 3320; MS (FAB, NBA matrix) *m/z* 1419 (MNa^+), 1398 (MH^+), 1353, 1263, 1150; UV (MeOH) 208 (ε = 41 582), 258 (ε = 609). Anal. ($C_{72}H_{104}N_{10}O_{18}$) C, H, N.

4-Nitrophenyl Bromoacetate. Dicyclohexylcarbodiimide (9.28 g, 45 mmol) was added to a stirred solution of 5.0 g (35.9 mmol) of bromoacetic acid and 8.50 g (61.1 mmol) of 4-nitrophenol in 180 mL of EtOAc at 0 °C. The mixture was stirred for 16 h at 5 °C, and 1 mL of acetic acid was added. The mixture was stirred for 20 min at room temperature and placed in the freezer for 20 min. The solid material was removed by filtration, and the filtrate was concentrated to a viscous oil and crystallized from Et_2O/hexanes to provide 7.73 g (83%) of 4-nitrophenyl bromoacetate as flakes: mp 86–87 °C (lit.[9] mp 88–91 °C); TLC R_f 0.63 (50/50/1 hexanes/EtOAc/HOAc); ^{1}H NMR ($CDCl_3$) δ 4.13 (s, 2H), 7.36 (d, J = 12 Hz, 2H), 8.32 (d, J = 12 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 24.9, 122.1, 125.3, 155.5, 164.9; IR (KBr pellet, cm^{-1}) 1770, 2863, 3020, 3074, 3111. Anal. ($C_8H_6BrNO_4$) C, H, N.

1,2-Ethanediylbis(oxy-2,1-ethanediylbis[2-[[6-[(bromoacetyl)amino]-1-oxohexyl]amino]ethyl]carbamate), 3a. A solution of 8.0 g (5.7 mmol) of **2** in 50 mL of absolute EtOH and 35 mL of cyclohexene was placed under nitrogen, and 500 mg of 10% Pd on carbon was added. The mixture was refluxed with stirring for 2 h. When cool, the mixture was filtered through Celite and concentrated to give 5.0 g of an oil. The CBZ protecting groups were completely removed as evidenced by NMR: ^{1}H NMR (50/50 $CDCl_3$/CD_3OD) d 1.21 (m, 8H), 1.49

(m, 8H), 1.62 (m, 8H), 2.19 (t, J = 7.4 Hz, 8H), 2.67 (t, J = 7.4 Hz, 8H), 3.36 (bd s, 16H), 3.67 (s, 4H), 3.71 (m, 4H), 4.21 (m, 4H).

The oil was dissolved in 37.5 mL of dioxane and 12.5 mL of H_2O containing 3.9 g (46.4 mmol) of $NaHCO_3$. The mixture was cooled to 0 °C in an ice bath, and 8.7 g (34.8 mmol) of 4-nitrophenyl bromoacetate was added. The mixture was stirred at 0 °C for 1 h, and 50 mL of 1 N H_2SO_4 was added slowly. The mixture was extracted with three 50 mL portions of EtOAc. The EtOAc extracts were discarded, and the aqueous layer was extracted with six 50 mL portions of 20/80 MeOH/CH_2Cl_2. The combined MeOH/CH_2Cl_2 layers were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by silica gel chromatography (step gradient, 9/1 CH_2Cl_2/MeOH and then 85/15/5 CH_2Cl_2/MeOH/THF) to provide 3.62 g (46%) of **3a** as a white solid: mp 87–89 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (m, 8H), 1.55 (m, 8H), 1.64 (m, 8H), 2.26 (m, 8H), 3.28 (m, 8H), 3.42 (bd s, 16H), 3.66 (s, 4H), 3.70 (m, 4H), 3.89 (s, 8H), 4.19 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 25.1, 26.2, 28.8, 29.0, 38.5, 39.1, 40.0, 47.8, 48.3, 64.7, 69.1, 70.3, 157.0, 166.3, 174.9; IR (KBr pellet, cm^{-1}) 1232, 1559, 1638, 1668, 1701, 2862, 2937, 3083, 3266; UV (MeOH) 206 (ϵ = 68 285); MS (FAB) *m/e* (rel intensity) MH$^+$ [1341 (25), 1343 (60), 1345 (70), 1347 (56), 1349 (21)], 705.6 (100). Anal. ($C_{48}H_{84}N_{10}O_{14}Br_4$) C, H, N.

Reduction of 4 to 5 and Reaction of 5 with 3a To Give Conjugate 6a. A solution of 82 500 OD_{260}[18] (3.11 g, 450 μmol) of 4 in 237 mL of helium-sparged pH 5 0.1 M sodium acetate was treated with 1.0 mL (814 mg, 4.30 mmol) of tributylphosphine for 1 h at room temperature with magnetic stirring. The reduced oligonucleotide **5** was precipitated by adding 385 mL of isopropyl alcohol. The mixture was kept at −20 °C for 1 h and centrifuged at 5 °C for 20 min at 6000 rcf. The supernatant was removed, and the pellet was dissolved in 275 mL of 0.3 M NaCl solution. A second precipitation with 385 mL of isopropyl alcohol was performed. The mixture was kept at −20 °C for 1 h and centrifuged at 5 °C for 20 min at 6000 rcf. The supernatant was removed, and the oily pellet was placed under vacuum for 16 h to give **5** as 3.91 g of foamy solid. The solid was dissolved in 49 mL of helium-sparged pH 10 0.1 M sodium borate buffer containing 1 mM EDTA. The mixture was kept under Ar, and a solution of 129 mg (96 μmol) of **3a** in 3.22 mL of 9/1 MeOH/H2O was added. The mixture was stirred for 20 h at room temperature and then kept at 5 °C. One-half of the mixture was purified by ion exchange chromatography on 300 mL of Fractogel (gradient, 0.5 M NaCl, 10% MeOH, pH 7.5 0.05 M sodium phosphate to 0.65 M NaCl, 10% MeOH, pH 7.5 0.05 M sodium phosphate). The fractions were combined which were pure as evidenced by HPLC on a NucleoPac column (1 mL/min, 260 nm; gradient, (A) 0.05 M pH 8.3 Tris, 20% CH_3CN, (B) 0.05 M pH 8.3 Tris, 1 M NaCl, 20% CH_3CN; 0–1 min, 5% B; 1–5 min, 5–15% B; 5–24 min, 15–70% B; t_R 19.2 min). The pure fractions were combined and concentrated on a Macro-Prep High Q column using 1.2 M NaCl, pH 12, to elute.[23] The concentrated material was precipitated by adding an equal volume of isopropyl alcohol and allowing to stand at −20 °C for 1 h. The mixture was centrifuged at 6000 rcf for 20 min. The pelleted precipitate was dialized against 0.5X sterile PBS to give 11 754 OD_{260}[18] (450 mg, 29%) of **6a** in a volume of 16 mL (735 OD_{260}/mL): DNA sequence by Maxam–Gilbert technique,[16,22] CACACACACACACACACACA; MS (electrospray) calcd for $C_{832}H_{1096}N_{330}O_{458}P_{80}S_4$, 25 641; found, 25 720.[17]

A buffer-free analytical sample was prepared by lyophilizing and then dialyzing the resulting powdery solid extensively with water. The dialysate was lyophilized to a powder. Anal. ($C_{832}H_{1016}N_{330}O_{458}P_{80}Na_{80}S_4$) C, N, P.

Annealing of 6a with (TG)$_{10}$: Preparation of LJP 394. A mixture of 5489 OD_{260} (210 mg, 7.6 μmol) of **6a** in a volume of 7.7 mL of 0.5× PBS and a mixture 5842 OD_{260} (214 mg, 32 μmol) of $(TG)_{10}$ in 1.94 mL of H_2O were mixed and heated at 70 °C in a water bath for 5 min. The bath was allowed to cool to room temperature, and the mixture was analyzed for $(TG)_{10}$ by HPLC (Shodex; 1 mL/min, 260 nm; isocratic, 0.1 M sodium phosphate, pH 7.5, 1 mM EDTA, 7.5% CH_3CN; t_R for **7a**, 6.9 min, t_R for $(TG)_{10}$, 9.0 min). No excess $(TG)_{10}$ was observed, so an additional 430 OD_{260} (16 mg, 2.3 μmol) of $(TG)_{10}$ was added. The mixture was again heated to 70 °C and allowed to cool slowly to room temperature. Analysis by HPLC (Shodex) showed a 6% excess of $(TG)_{10}$. The solution of LJP 394 contained 9104 OD_{260}[18] (approximately 7.5 μmol, 99%), T_m 67.1 °C, hyperchromicity 25.6% (0.14 M NaCl, 0.01 M sodium cacodylate, pH 6.8). A representative melt curve is presented in Figure 6.

An analytical sample was further purified by HPLC (ProteinPac Q-HR15 packing in a 2 cm × 10 cm column, 5 mL/min; gradient, (A) 50 mM sodium phosphate, 1 mM EDTA, 5% CH_3CN, pH 7.5, (B) 50 mM sodium phosphate, 1 mM EDTA, 5% CH_3CN, 1 M NaCl, pH 7.5; 30–70% B). Pure fractions were lyophilized and then dialyzed extensively with water. The dialysate was lyophilized to a powdery solid. Anal. ($C_{1632}H_{1944}N_{610}O_{970}Na_{156}P_{156}S_4$) C, N, P.

Preparation of ^{14}C Analogues 6b and 7b. This preparation was essentially identical to the **6a** preparation with the exception that bromoacetic acid was replaced with 2-bromo-[1-^{14}C]acetic acid (Amersham; 53 mCi/mmol) which was used to make 4-nitrophenyl 2-bromo[1-^{14}C]acetate.[10] The labeled 4-nitrophenyl ester was in turn used to make **3b**. Conjugation of 4 equiv of **5** with **3b** gave **6b**. The radiolabeled conjugate **6b** was annealed with $(TG)_{10}$ to provide double-stranded conjugate **7b**.

Immunization and Tolerization of Mice. Female C57Bl/6 mice, 6–8 weeks of age, were obtained from Jackson Laboratories and housed in accordance with NIH guidelines. The mice were primed as previously described.[24] Briefly, mice were injected, ip, with 100 μg of double-stranded oligonucleotide-KLH conjugate[4] ([TG_{25}·CA_{25}]$_5$-KLH) precipitated on alum (aluminum hydroxide gel) along with 2 × 10^9 *Bordetella pertussis* organisms (obtained from Massachusetts Department of Public Health, State Laboratory Institute, Boston, MA) as an adjuvant. Three weeks after priming, the mice were divided into groups of three and injected, ip, with graded doses of LJP 394. One group was treated with saline and served as the nontreated control. Five days after treatment with the toleragen LJP 394, all of the mice, including the controls, were boosted with 50 μg of [TG_{25}·CA_{25}]$_5$-KLH. The mice were sacrificed 4 days after the boost.

Biological Activity Assays. Plaque-Forming Cell Assay. Spleens were analyzed for the number of IgG anti-ds-ON antibody-forming cells using a standard indirect plaque-forming cell assay in which indicator cells were prepared by coupling a conjugate of double-stranded oligonucleotide and D-EK (ds-ON-D-EK)[4] to sheep red blood cells (SRBC) using EDCI.[25] The concentration of ds-ON-D-EK used was determined experimentally to be that which did not cause spontaneous agglutination or lysis of the SRBC or agglutination of SRBC in the presence of mouse anti-ds-ON antiserum but did cause agglutination of SRBC in the presence of both mouse anti-ds-ON antiserum and rabbit anti-mouse immunoglobulin.

Spleen cells, from individual mice, were mixed with indicator cells, rabbit anti-mouse immunoglobulin, and guinea pig serum, which serves as a source of complement. The concentration of rabbit anti-mouse immunoglobulin used was determined experimentally to be that which completely inhibited the development of all IgM plaques and developed the maximum number of IgG plaques. The mixture was placed in a Cunningham chamber and incubated for 1 h at 37 °C. The number of plaques was counted by light microscopy. Each spleen cell preparation was also tested against SRBC that had not been coated with ds-ON-D-EK to determine the number of nonspecific plaques; they were always less than 1% of the number of specific plaques. The difference between the number of specific and nonspecific plaques is the number of ds-ON-specific IgG plaque-forming cells.

Farr Assay for Anti-ds-ON. Anti-ds-ON antibodies in serum were analyzed by the Farr assay[21] at a final antigen concentration of 10^{-8} M. A 5′-hydroxyphenyl-containing $(CA)_{25}$ oligonucleotide[26] was prepared and annealed with a complimentary $(TG)_{25}$ strand. The duplex was radiolabeled with ^{125}I as described by Fontanel[26] and used to determine antigen-binding capacity.[27]

ELISA Assay for Anti-KLH. A sandwich-type ELISA was used to measure anti-KLH antibodies as determined by interpolation from a standard curve generated using a stan-

dard pool of anti-KLH plasma. The measurement of anti-KLH antibodies is expressed as a percent of the standard pool.

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(5) The convention used herein to describe repeating dimers of deoxynucleotides is exemplified as follows. The 20-mer ON, $(CA)_{10}$, is an alternating sequence of deoxycytidine and deoxyadenosine with deoxycytidine at the 5′-end. The 50-mer ON, $(CA)_{25}$, is an alternating sequence of deoxycytidine and deoxyadenosine with deoxycytidine at the 5′-end. Similarly, $(TG)_{10}$ is a 20-mer ON with deoxythymidine at the 5′-end, and $(TG)_{25}$ is a 50-mer ON with deoxythymidine at the 5′-end.

(6) Experiments with different sequences of synthetic oligonucleotide duplexes demonstrated sequence dependence on binding to anti-ds-DNA from lupus mice and humans, as detected by competitive inhibition assays with high molecular weight DNA. This was apparently related to the stability of the duplex and its propensity to assume the B DNA conformation. The duplex formed from $(CA)_{25}$ and $(TG)_{25}$, under physiological conditions, assumes the B DNA conformation as evidenced by CD spectroscopy.

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(13) Precipitation of the thiol-containing oligonucleotide removes tributylphosphine, tributylphosphine oxide, and *O*-trityl-6-mercaptohexan-1-ol, the other half of the disulfide.

(14) HPLC analyses were carried out using a GenPak FAX column (1 mL/min, gradient, 30–70% B (A = 0.05 M pH 7.5 sodium phosphate in 10% MeOH, B = 0.05 M pH 7.5 sodium phosphate and 1.0 M NaCl in 10% MeOH) 0–16 min, 260 nm).

(15) The reactions were run in 100 mM pH 10 sodium borate buffer at 25 °C. The concentration of **5** was kept at 25 mg/mL, and stoichiometry was adjusted by adding aliquots of **3** from a 40 mg/mL solution in 9/1 MeOH/H_2O.

(16) Sequencing was done by a modification of the method of Maxam and Gilbert,[22] using 3′-end labeling with terminal transferase and [^{32}P]dideoxy-ATP. Thus 1 μL of oligonucleotide-containing solution (5.28 μg), 4 μL of 5X terminal transferase buffer (Promega), 5 μL of [^{32}P]dideoxy-ATP (Amersham), 9 μL of H_2O, and 1 μL of terminal transferase solution (Amersham) were incubated for 30 min at 37 °C. The mixture was desalted on a PD-10 gel filtration column (Parmacia). Fractions containing oligonucleotide were concentrated to dryness and dissolved in 50 μL of H_2O. Chemical cleavage was performed on 10 μL of the mixture using standard Maxam–Gilbert techniques. The cleavage mixtures were analyzed on a 20% polyacrylamide gel.

(17) An oligonucleotide of this size is near the limit for routine application of the electrospray technique. The mass spectrum of **6a** was obtained from the Mass Spectroscopy Lab at the Scripps Institute, La Jolla, CA, using a API III Perkin Elmer Sciex triple-quadrupole mass spectrometer with a Tune data system and MacSpec reconstruct program to calculate the molecular weight. A sodium-free sample was prepared for mass spectrometry by dialyzing extensively with 0.1 M pH 6.5 triethylammonium borate followed by dialysis with water. The dialysate was lyophilized to a solid. Reconstruction of molecular ion peaks from fragments showed a broad series of peaks centered at 25 720 mass units, with a width at half-height of 130 mass units.

(18) Quantitation of oligonucleotides was conveniently determined by measuring optical density at 260 nm (OD_{260}). Absorbances were measured on dilute solutions which had an absorbance less than 1.0 in pH 7.2 PBS. Optical density was determined by multiplying absorbance by the dilution factor. The molar extinction coefficient for **6a** at 260 nm was determined by three methods: (1) phosphorus determination assuming 80 mol of phosphorus for 1 mol of **6a**, (2) carbon combustion analysis assuming a chemical formula of $C_{832}H_{1016}N_{330}O_{458}P_{80}Na_{80}S_4$, and (3) determination of specific activity for ^{14}C-labeled **6b** assuming the specific activity of **6b** to be 212 mCi/mmol (4 times the specific activity of the ^{14}C-labeled bromoacetic acid used in its preparation). The values determined by the three methods were in close agreement (within 3%), with the mean being 715 668 ± 15 096 M^{-1} cm^{-1} (26.11 ± 0.55 mL mg^{-1} cm^{-1}). The molar extinction coefficient for **7a** was determined by three methods: (1) phosphorus determination assuming 156 mol of phosphorus for 1 mol of LJP 394, (2) carbon combustion analysis assuming a chemical formula of $C_{1632}H_{1944}N_{610}O_{970}P_{156}S_4$, and (3) determination of specific activity for ^{14}C-labeled **7b** assuming the specific activity of **7b** to be 212 mCi/mmol. The values determined by the three methods were in close agreement, with the mean being 1 208 962 ± 26 971 M^{-1} cm^{-1} (22.25 ± 0.56 mL mg^{-1} cm^{-1}).

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(23) It is important to use highly purified **4** in the conjugation process because any reactive impurity becomes amplified by a factor of 4, manifested as an impurity in the tetrasubstituted conjugate **6a**. For example, a 5% impurity in **4**, if reactive, would produce a 20% impurity in **6a** Analytical reversed phase HPLC was performed on fractions collected in the anion exchange column purification of **4**, and only fractions of >90% purity were used. This resulted in a final purity of 95%.

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JM9408530