

## Electronic Supplementary Information

### Amphipathic *trans*-acting phosphorothioate DNA elements mediate the delivery of uncharged nucleic acid sequences in mammalian cells

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**Materials and methods.** Common chemicals and solvents including acetonitrile, benzene, triethylamine, dichloromethane, hexane, acetone, DMSO, 3-(*N,N*-dimethylamino)propan-1-ol, 1-octanol, *bis*(*N,N*-diisopropylamino)chlorophosphine, anhydrous solvents (MeCN, CH<sub>2</sub>Cl<sub>2</sub>, C<sub>6</sub>H<sub>6</sub>) and deuterated solvents (C<sub>6</sub>D<sub>6</sub>, DMSO-*d*<sub>6</sub>) were all purchased from commercial sources (Fisher or Aldrich) and used without further purification.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-deoxythymidine and *N*<sup>6</sup>-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine were purchased from ChemGenes and were used as received. All ancillary reagents commonly used in solid-phase DNA/RNA synthesis including 3*H*-1,2-benzodithiol-3-one 1,1-dioxide, 3-(dimethylaminomethylidene)amino-3*H*-1,2,4-dithiazole-3-thione and succinylated long chain alkylamine controlled-pore glass (CPG) support functionalized with either 2'-deoxythymidine or *N*<sup>6</sup>-benzoyl-2'-deoxyadenosine, as the leader nucleoside, were obtained from Glen Research and used without further purification.

The PNA and PMO oligomers listed in Table 1 or mentioned anywhere in the text were purchased from either PNA Bio Inc. or Gene Tools LLC and used as received.

Flash chromatography purifications were performed on glass columns (6.0 cm or 2.5 cm I.D.) packed with silica gel 60 (EMD, 230-400 mesh), whereas analytical thin-layer chromatography (TLC) analyses were conducted on 2.5 cm × 7.5 cm glass plates coated with a 0.25 mm thick layer of silica gel 60 F<sub>254</sub> (EMD).

Analytical RP-HPLC analyses of dTtaPS and dAtaPS were performed using a 5 μm Vydac C-4 wide pore (300 Å) column (25 cm × 4.6 mm). A linear gradient running from MeCN:H<sub>2</sub>O:AcOH (199:799:2, v/v/v to 799:199:2, v/v/v) at a flow rate of 1 ml/min for 20 min was conducted and found adequate for its intended purpose.

<sup>1</sup>H-Decoupled <sup>31</sup>P NMR analysis of the deoxyribonucleoside phosphoramidites **3-6** was performed using an NMR spectrometer operating at 121.5 MHz (300 MHz for <sup>1</sup>H). Samples were maintained at a temperature of 298°K; all spectra were recorded in deuterated solvents and chemical shifts δ were reported in parts per million (ppm) relative to appropriate internal references.

High resolution mass spectra of the deoxyribonucleoside phosphoramidites **3-6** were obtained using a Bruker Daltonics ApexQ FT-ICR mass spectrometer equipped with a 12 T magnet. Electrospray ionization in positive ion mode was used to generate [M+H]<sup>+</sup> and [M+Na]<sup>+</sup> ions out of test samples [0.01 mg dissolved in 1 mL of 10 mM ammonium acetate in MeCN:H<sub>2</sub>O (1:1 v/v)]. Spectra were externally calibrated using 0.5 mg/mL solution of CsI in water, which yielded a series of peaks in the mass range used for analysis (200-2000 m/z).

MALDI-TOF mass spectrometry; analyses were performed on a Voyager-DE RP instrument operating in a delayed extraction reflector mode using a matrix composed of 2,4,6-triacetoxyacetophenone (10 mg/mL in 50% aqueous MeCN) and ammonium citrate (50 mg/mL in H<sub>2</sub>O) in a ratio of 1:1 (v/v). Each analyte was prepared by premixing 1 μL of the DNA sequence solution (1 OD<sub>260</sub> dissolved in 10 μL of 50% aqueous MeCN) with 1 μL of the matrix solution. The analytes were deposited on a stainless steel plate and allowed to dry at ambient temperature. The duplicate analyte was exclusively dedicated to mass determination within 0.2% of the theoretical mass under optimized instrumental conditions.

### ***N,N,N',N'*-Tetraisopropyl-*O*-[3-(*N,N*-dimethylamino)prop-1-yl]phosphordiamidite (1)**

To a stirred solution of 3-(*N,N*-dimethylamino)propan-1-ol (436  $\mu$ L, 3.70 mmol) and *bis*(*N,N*-diisopropylamino)chlorophosphine (1.00 g, 3.70 mmol) in anhydrous benzene (20 ml) was added, under inert atmosphere, Et<sub>3</sub>N (1.00 ml, 7.17 mmol). Progress of the reaction was monitored by <sup>31</sup>P-NMR spectroscopy; formation of the product ( $\delta_p$  122.4 ppm) was found to be complete within 2 h at ~25°C. The suspension was passed through a glass column packed with silica gel (~15 g), which had been pre-equilibrated in benzene:Et<sub>3</sub>N (9:1 v/v); the filtrate was evaporated to an oil under reduced pressure. The oily material was dissolved in dry benzene (10 ml) and the resulting solution was swirled in a dry ice-acetone bath. The frozen material was lyophilized under high vacuum to give the phosphordiamidite as a viscous oil (1.10 g, 3.29 mmol, 88%), which was of sufficient purity to be used without further purification in the preparation of deoxyribonucleoside phosphoramidites **3** and **5**.

### ***N,N,N',N'*-Tetraisopropyl-*O*-[octan-1-yl]phosphordiamidite (2)**

The preparation of this compound was performed at the same scale and under conditions similar to those employed for the synthesis of **1** with the exception of using 1-octanol (583  $\mu$ l, 3.70 mmol) instead of 3-(*N,N*-dimethylamino)propan-1-ol. The phosphordiamidite **2** was isolated as a viscous oil, the yield of which was similar to that obtained for **1**, after silica gel purification. The phosphordiamidite was used without further purification in the preparation of deoxyribonucleoside phosphoramidites **4** and **6**.

### **General procedure for the preparation of deoxyribonucleoside phosphoramidites (3-6)**

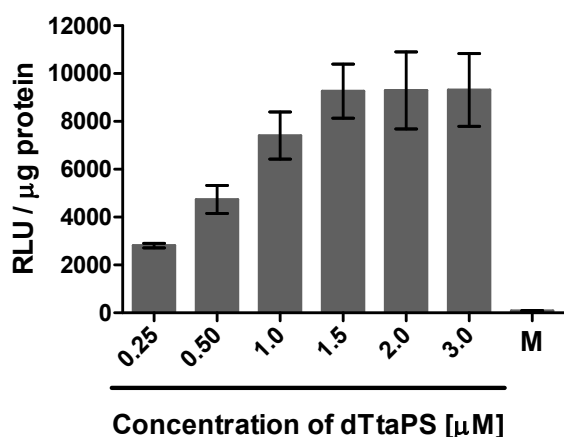
To a stirred solution of *N,N,N',N'*-tetraisopropyl-*O*-[3-(*N,N*-dimethylamino)prop-1-yl]phosphordiamidite or *N,N,N',N'*-tetraisopropyl-*O*-[octan-1-yl]phosphordiamidite (2.0 mmol) in MeCN (20 ml) was added either 4,4'-dimethoxytrityl deoxythymidine or *N*<sup>6</sup>-benzoyl-4,4'-dimethoxytrityl 2'-deoxyadenosine (1.0 mmol) along with 0.45 M 1*H*-tetrazole in MeCN (2.2 ml, 1.0 mmol). The reaction mixture was stirred for 18 h at ~25°C. The reaction mixture was then concentrated under reduced pressure to a gummy material; the crude phosphoramidite was purified by chromatography on silica gel (~25 g), which was equilibrated in a solution of hexane:Et<sub>3</sub>N (95:5 v/v). The product was eluted from the column using a gradient of CH<sub>2</sub>Cl<sub>2</sub> (0 → 95%) in hexane:Et<sub>3</sub>N (95:5 v/v). Fractions containing the pure phosphoramidite, as indicated by TLC, were pooled together and were evaporated to dryness under low pressure. The foamy material was dissolved in dry benzene (5 ml) and the resulting solution was manually stirred in a dry ice-acetone bath. The frozen material was then lyophilized under high vacuum to give the phosphoramidites **3-6**, each as a white powder. The isolated yield of each deoxyribonucleoside phosphoramidite was in the range of (75-85%).

**5'-*O*-(4,4'-Dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(3-[*N,N*-dimethylamino]prop-1-yl)oxy]phosphinyl-2'-deoxythymidine (3).** <sup>31</sup>P NMR (121 MHz, C<sub>6</sub>D<sub>6</sub>):  $\delta$  148.8, 148.3. +HRMS: calcd for C<sub>42</sub>H<sub>57</sub>N<sub>4</sub>O<sub>8</sub>P [M]<sup>+</sup> 777.3986, found 777.3984.

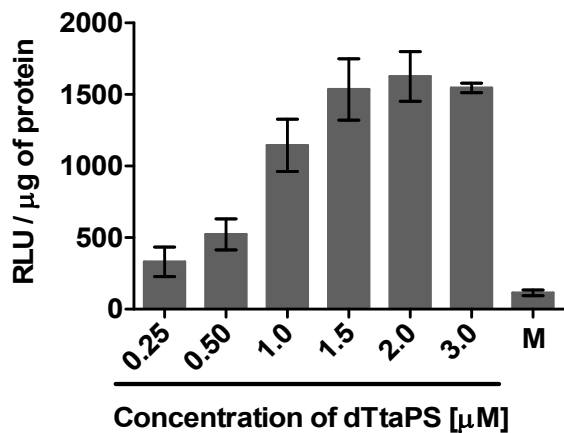
**5'-*O*-(4,4'-Dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(octan-1-yl)oxy]phosphinyl-2'-deoxythymidine (4).** <sup>31</sup>P NMR (121 MHz, C<sub>6</sub>D<sub>6</sub>):  $\delta$  147.7, 147.0. +HRMS: calcd for C<sub>45</sub>H<sub>62</sub>N<sub>3</sub>O<sub>8</sub>P [M + H]<sup>+</sup> 804.4353, found 804.4338. ***N*<sup>6</sup>-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(3-[*N,N*-dimethylamino]prop-1-yl)oxy]phosphinyl-2'-deoxyadenosine (5).** <sup>31</sup>P NMR (121 MHz, C<sub>6</sub>D<sub>6</sub>):  $\delta$  148.6. +HRMS: calcd for C<sub>49</sub>H<sub>60</sub>N<sub>7</sub>O<sub>7</sub>P [M + H]<sup>+</sup> 890.4364, found 890.4353. ***N*<sup>6</sup>-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(octan-1-yl)oxy]phosphinyl-2'-deoxyadenosine (6).** <sup>31</sup>P NMR (121 MHz, C<sub>6</sub>D<sub>6</sub>):  $\delta$  148.4, 148.3. +HRMS: calcd for C<sub>52</sub>H<sub>65</sub>N<sub>6</sub>O<sub>7</sub>P [M + H]<sup>+</sup> 917.4725, found 917.4767.

Cell culture reagents including Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, sodium pyruvate, penicillin, streptomycin, hygromycin, Lipofectamine™ 2000 and Trypsin, were obtained from Invitrogen (Life Technologies). Chlorpromazine hydrochloride, Nystatin and 5-(*N*-ethyl-*N*-isopropyl)amiloride were purchased from Sigma-Aldrich and used as received. Phenol red MEM medium, Phenol red-free DMEM medium and 0.4% Trypan Blue were obtained from MediaTech. Monensin (Sigma) was kindly provided by Dr. James Keller (CBER, FDA).

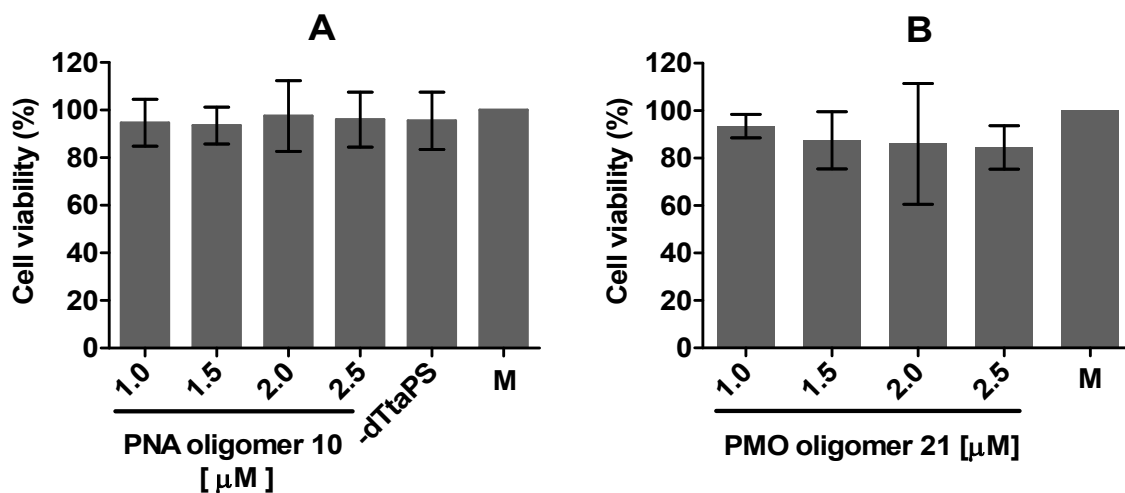
Pierce Coomassie (Bradford) reagent for protein concentration measurement and TRIzol reagent for RT-PCR analyses were purchased from Life Technologies, Carlsbad, CA. High capacity cDNA Reverse Transcription Kit and 2x SYBR green were purchased from Applied Biosystems, Foster City, CA. The CCK-8 Kit for cytotoxicity studies was purchased from Dojindo Molecular Technologies. Flat-bottom 96-well plates were purchased from BD-Falcon.



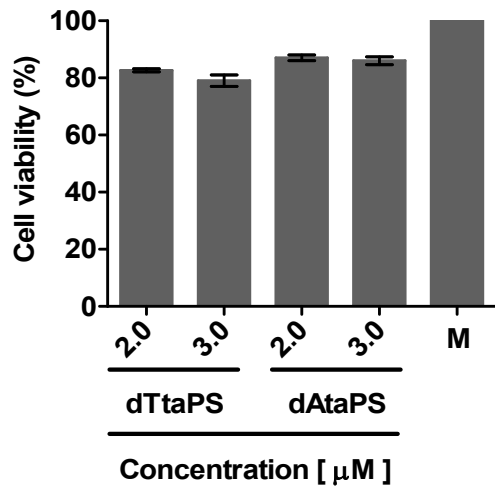
**Fig. S1** Relevance of the concentration of dTtaPS to the production of luminescence upon transfection of the polyA-tailed PNA oligomer **10** (1.0 μM) into HeLa pLuc705 cells. Error bars represent the mean ± SD of three independent experiments. M, medium



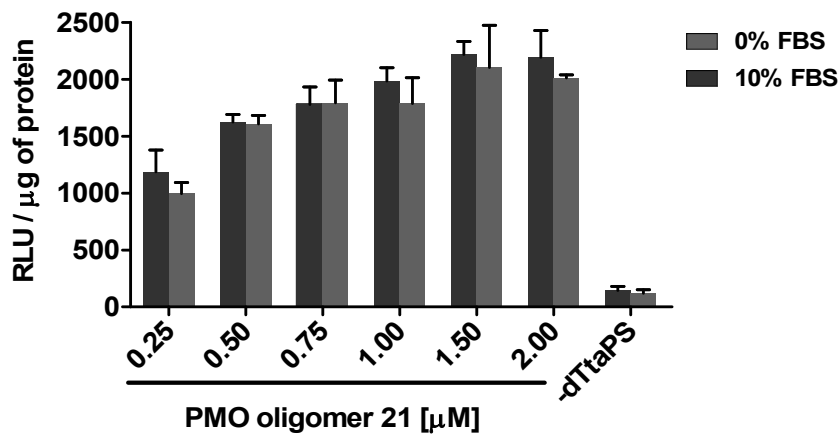
**Fig. S2** Relevance of the concentration of dTtaPS to the production of luminescence upon transfection of the polyA-tailed PMO oligomer **21** ( $1.0 \mu\text{M}$ ) into HeLa pLuc705 cells. Error bars represent the mean  $\pm$  SD of three independent experiments. M, medium



**Fig. S3** (A) Cytotoxicity of PNA oligomer **10**:dTtaPS and (B) PMO oligomer **21**:dTtaPS complexes in HeLa pLuc 705 cells. The dTtaPS concentration is kept at  $2.0 \mu\text{M}$ . Error bars represent the mean  $\pm$  SD. M, medium.

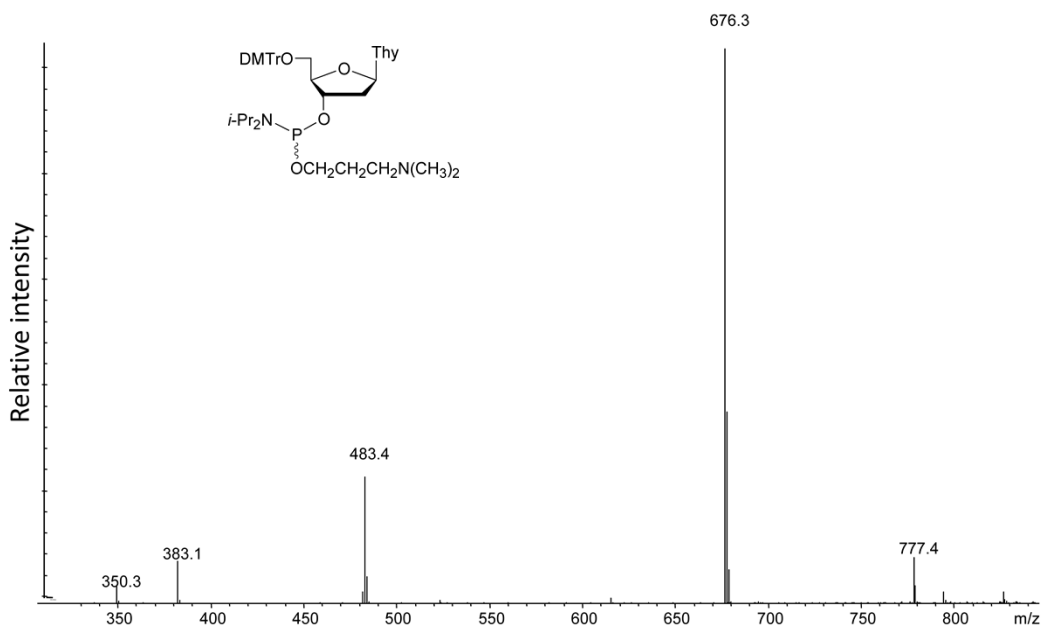


**Fig. S4** Cytotoxicity of dTtaPS and dAtaPS in HeLa pLuc705 cells at concentrations for optimal transfection efficiency. Error bars represent the mean  $\pm$  SD. The cytotoxicity study was performed using a CCK-8 kit as per the manufacturer's recommendations. M, medium

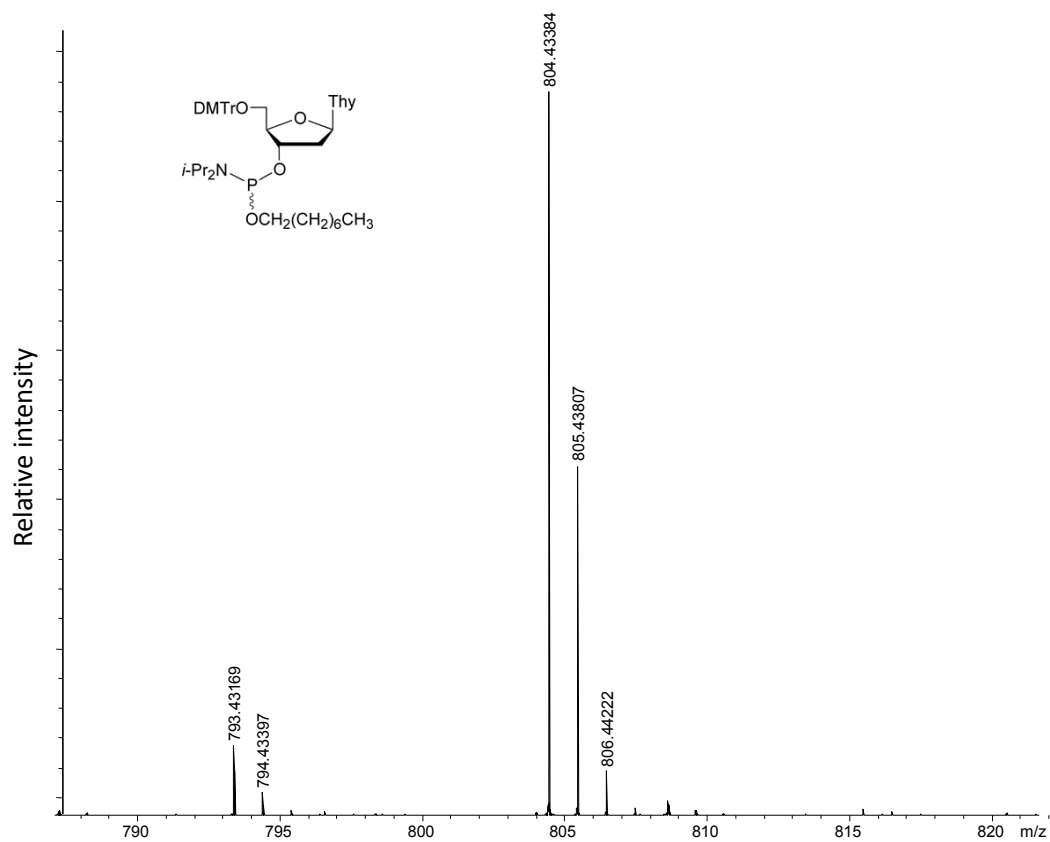


**Fig. S5** Concentration-dependence of the dTtaPS-mediated delivery of the PMO oligomer **21** in the restoration of luciferase activity in serum-containing and serum-free media. The dTtaPS concentration is kept at 2.0 μM. Error bars represent the mean  $\pm$  SD.

**HRMS spectrum of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-[(*N,N*-diisopropylamino)(3-[*N,N*-dimethylamino]prop-1-yl)oxy]phosphinyl-2'-deoxythymidine (3)**

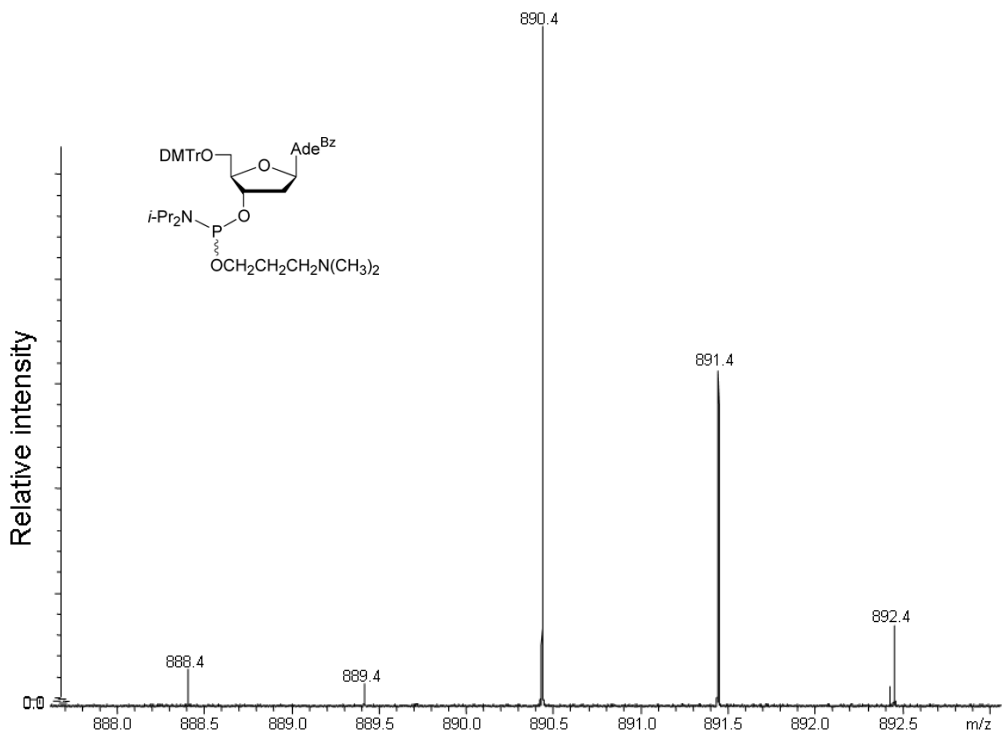


**HRMS spectrum of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-[(*N,N*-diisopropylamino)(octan-1-yl)oxy]phosphinyl-2'-deoxythymidine (4)**





**HRMS spectrum of  $N^6$ -Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(3-[*N,N*-dimethylamino]prop-1-yl)oxy]phosphinyl-2'-deoxyadenosine (5)**



**HRMS mass spectrum of *N*<sup>6</sup>-Benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-[(*N,N*-diisopropylamino)(octan-1-yl)oxy]phosphinyl-2'-deoxyadenosine (6)**

