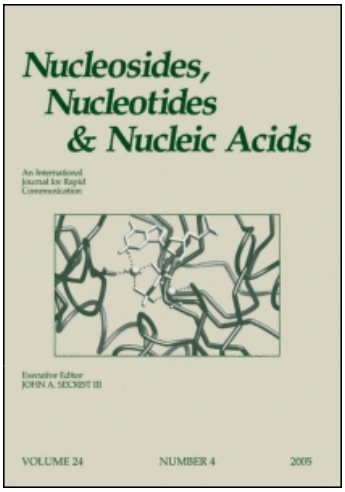

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Dmitry A. Stetsenko[a]; Michael J. Gait
[a] Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK

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NEW PHOSPHORAMIDITE REAGENTS FOR THE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING A CYSTEINE RESIDUE USEFUL IN PEPTIDE CONJUGATION [1]

Dmitry A. Stetsenko and Michael J. Gait *

Medical Research Council, Laboratory of Molecular Biology, Hills Road, CB2 2QH Cambridge, UK

ABSTRACT: The preparation is described of four 2–cyanoethyl-*N*,*N*-diisopropyl phosphoramidites of *N*-α-Fmoc-*S*-protected cysteine hydroxyalkyl amides. The phosphoramidites were used in solid-phase synthesis of 5'-cysteinyl oligonucleotides, useful intermediates in the preparation of peptide-oligonucleotide conjugates through reaction with a maleimide peptide or with a peptide thioester *via* "native ligation".

INTRODUCTION

Synthetic oligonucleotides and their derivatives have found wide application as potential therapeutic agents through their ability to inhibit the expression of target genes sequence-selectively.[1-2] However, efficient intracellular delivery of oligonucleotide analogues has remained a significant challenge, especially into cells in culture. Cellular uptake of oligonucleotides and analogues can be enhanced significantly by the co-administration of certain reagents such as cationic liposomes[3] or peptides.[4-7] Another approach that is being explored currently is the covalent attachment to the oligonucleotide of peptide transporters,[8-12] including some that have been shown to translocate through cell membranes apparently by a non-receptor mediated pathway.[13-16]

[1] This paper is dedicated to the memory of Professor Aleksandr Antonovich Krayevsky, a truly outstanding figure in contemporary Russian bio-organic chemistry.

* Tel: +44 (0)1223 248000, Fax: +44 (0)1223 402070, Email: mgait@mrc-lmb.cam.ac.uk

There are few available methods for peptide-oligonucleotide conjugate synthesis that are readily applicable to a large range of peptide and oligonucleotide sequences. A total stepwise solid-phase approach on a single support is the most ambitious but poses serious problems, mainly because of protecting group incompatibility. Therefore, this route has been limited so far to relatively short peptides or those lacking side-chain protection.[17-22] A more generally applicable strategy for peptide-oligonucleotide conjugation consists of separate solid-phase assembly of peptide and oligonucleotide sequences and their subsequent chemo-selective joining in solution[8,23-25] or on solid phase.[26] One popular conjugation chemistry is the reaction of a maleimide functionality with a thiol group. For example, a 5'-thiol-functionalised oligonucleotide may be reacted with a maleimide peptide.[27] Alternatively, a cysteine-containing peptide is reacted with a maleimide-functionalised oligonucleotide.[28,29] However, these routes tend to be cumbersome and usually involve some post-assembly manipulation of the modified peptide and oligonucleotide precursors. Recently, maleimide peptides have been coupled to oligonucleotides containing a C-terminal cysteine, introduced through synthesis of a cysteinyl linker attached to a specially prepared solid support.[30]

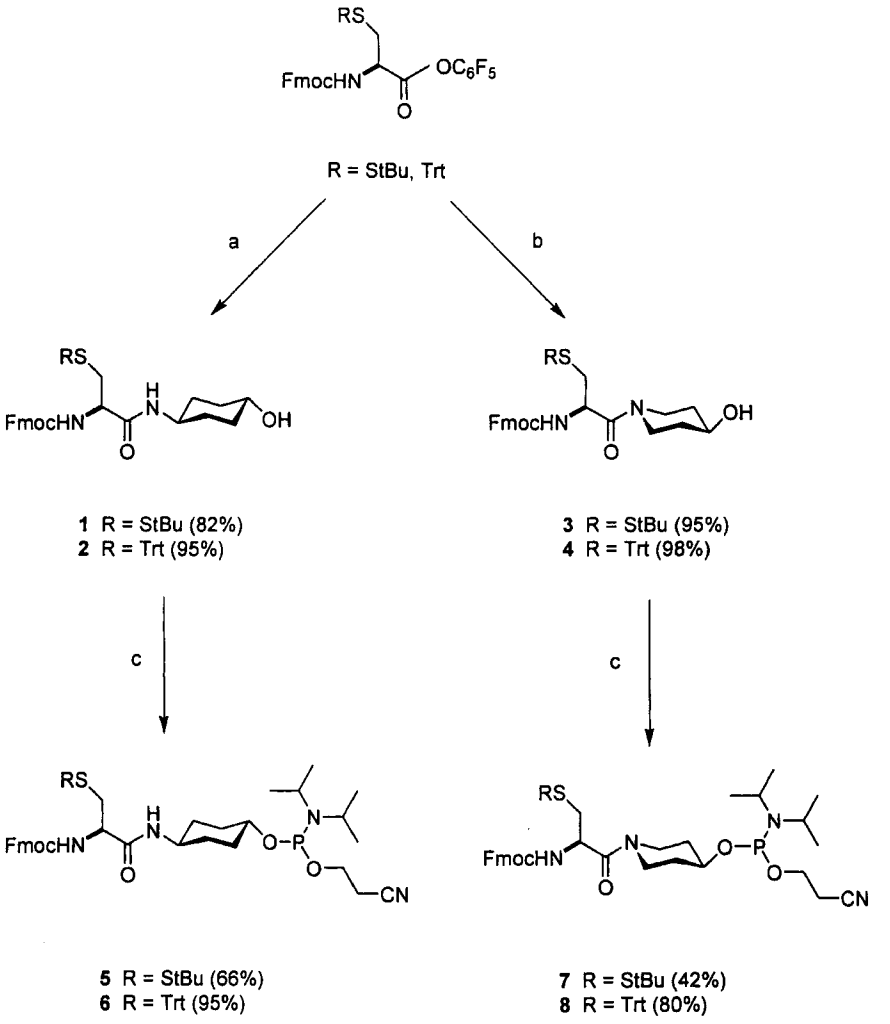
Since the 3'-ends of oligonucleotide analogues used for antisense studies are often used for attachment of fluorescent and other reporter groups, we decided to develop an alternative approach for the incorporation of a cysteine residue at the 5'-end of an oligonucleotide. We have therefore designed suitable phosphoramidite reagents that can be used in a final standard coupling step in oligonucleotide assembly by phosphoramidite synthetic methods. After deprotection, the cysteine moiety, now attached to the 5'-position of the oligonucleotide, provides a suitable thiol group for maleimide peptide attachment. Further, the cysteine phosphoramidite reagents are useful in a new approach to peptide-oligonucleotide conjugation based on a powerful "native ligation" approach[35].

RESULTS AND DISCUSSION

Instead of the more common primary alcohols used in preparation of many 5'-linker phosphoramidite reagents, we decided to use two alternative secondary amino alcohols, 4-*trans*-aminocyclohexanol and 4-hydroxypiperidine. Our choice was prompted by generally higher stability and longer shelf-life, and yet sufficiently high reactivity of the phosphoramidites derived from secondary alcohols. These were reacted with

commercially available *N*-α-Fmoc-*S*-protected cysteine pentafluorophenyl esters to give the corresponding amide derivatives **1-4** (**Scheme 1**). Subsequent phosphitylation of the secondary alcohol by standard methods[31] afforded the corresponding phosphoramidites **5-8**. Two alternative, orthogonal thiol protecting groups, trityl and *S*-*tert*-butylsulfenyl, were utilised in these syntheses as well as the common 9-fluorenylmethoxycarbonyl (Fmoc) as the amino protecting group. The thiol protecting groups can be removed selectively under mild conditions: brief silver nitrate solution treatment followed by dithiothreitol (DTT) for trityl[32] and *tris*-(carboxyethyl)phosphine (TCEP) reduction for *S*-*tert*-butylsulfenyl.[33] The yields of the trityl-protected phosphoramidites were generally higher than those protected by *S*-*tert*-butyl (**Scheme 1**), possibly due to some loss of thiol protecting group during work-up or chromatography. However, all four phosphoramidites were found to be white solids which could be stored for several months at –20 °C without loss of reactivity.

The reactivities of these cysteine phosphoramidites that are derived from secondary alcohols were found to be very similar to those prepared from regular nucleosides when used in standard oligonucleotide assembly by the phosphoramidite method.[34] For example, phosphoramidites **5** and **6** gave coupling yields of >97% as judged by HPLC when incorporated into a range of oligonucleotide products.[35] Both thiol protecting groups are stable during oligonucleotide synthesis under standard assembly and deprotection conditions. As an example of the efficiency of cysteine incorporation we synthesised a 3'-fluoresceinyl 15-mer oligodeoxyribonucleotide dCTCCCAGGCTCAAAT on 1 μmole scale using a commercially available fluorescein-derivatised controlled pore glass support. Then the cysteinylpiperidine phosphoramidite **7** was coupled and, after standard iodine oxidation, the terminal Fmoc group was removed by brief treatment with piperidine. Following standard aqueous ammonia deprotection and release into solution, the resultant 5'-*S*-*tert*-butylthiocysteine 3'-fluorescein oligonucleotide derivative showed by reversed phase HPLC a single main peak at 18.3 minutes elution time (**Figure 1**). After HPLC purification, the yield was 20.7 A_{260} units. The product showed a single peak by HPLC and a single band by polyacrylamide gel electrophoresis (data not shown). The MALDI-TOF mass spectra showed a mass of 5424 Da (**Figure 1 inset**)(calculated 5422). When this oligonucleotide was treated with 0.2 M TCEP solution (pH 6.5) for 30 min at room temperature, the free thiol was obtained



Key: (a) *trans*-4-aminocyclohexanol hydrochloride, NEt_3, DMF; (b) 4-hydroxypiperidine, NEt_3, DMF; c) 2-cyanoethoxy-*N*,*N*-diisopropylamino chlorophosphine, DIEA, CH_2Cl_2 (**5**, **8**), or 2-cyanoethyl *N*,*N*,*N'*,*N'*-tetraisopropyl phosphordiamidite, diisopropylammonium tetrazolide, CH_2Cl_2 (**6**, **7**).

SCHEME 1. Synthesis of cysteine phosphoramidites **5-8**.

quantitatively as observed by HPLC (elution time 16.7 min, data not shown). Preliminary experiments in conjugation reaction of this 5'-cysteine-3'-fluorescein oligodeoxynucleotide with tenfold excess of ε-(N-maleimido)hexanoyl-PKKKRKV peptide incorporating SV40 large T antigen NLS, in aqueous solution by method similar

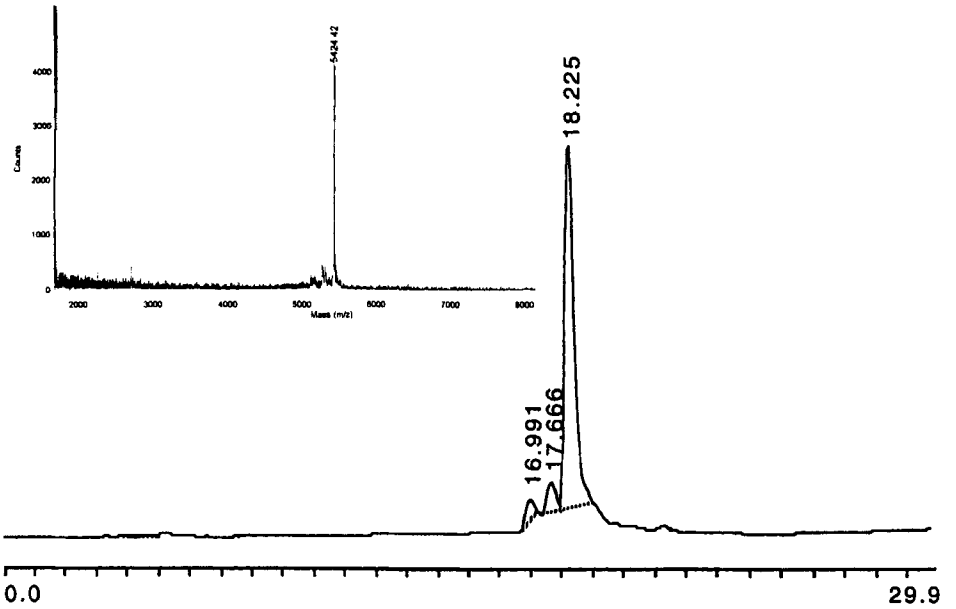


Figure 1. HPLC of the crude reaction product of the synthesis of 5'-*S-tert*-butylthiocysteine-3'-fluorescein derivative of oligodeoxyribonucleotide dCTCCCAGGCTCAAAT. Inset: MALDI-TOF mass spectrum of the same oligonucleotide product.

to that previously reported[30] showed that the oligonucleotide was completely consumed within 16 h. The fluorescent peptide-oligonucleotide conjugate was separated as the major product by denaturing PAGE and characterised by MALDI-TOF mass spectroscopy ($[M+H]^+$ found 6416.3, calculated 6410.0). Further characterisation and use of such peptide-oligonucleotide conjugates as antisense agents will be reported in due course.

We have also used phosphoramidite **5** in solid phase synthesis of 5'-cysteinyl oligodeoxyribonucleotides which were subsequently conjugated chemo-selectively in aqueous solution to an N-terminal peptide thioester in a new method of "native ligation"[35], based on the well-known methods previously developed for synthesis of proteins.[36-37] In principle, such 5'-cysteinyl oligonucleotides also should be able to be conjugated by the same techniques to C-terminal peptide thioesters prepared by

Boc/benzyl[38] or Fmoc/*tert*-butyl chemistry.[39,40] It should be noted that 5'-cysteine-substituted oligonucleotides should also be useful for conjugations to non-radioactive reporters such as fluorescent groups.

Experimental

General Methods. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. *N*-α-Fmoc cysteine derivatives were purchased from Novabiochem and Millipore. DMF (Fisher) was distilled *in vacuo* and used shortly after. Dichloromethane and acetonitrile (Fisher) were refluxed over CaH_2 followed by distillation. Other solvents were purchased from Fisher. Standard oligonucleotide synthesis reagents were purchased from PE Biosystems (ABI), Glen Research (via Cambio) and Cruachem (Scotland). All other fine chemicals were supplied by Aldrich and Fluka. Thin layer chromatography was carried out on Merck 60 F_{254} aluminium-coated silica gel plates. TLC developing systems were the following: a) EtOAc (system A); b) EtOAc-hexane-NEt_3 (40:55:5 v/v/v) (system B); EtOAc-hexane-NEt_3 (30:65:5 v/v/v) (system C). Spots were visualised by UV exposure with a 254 nm lamp. Column chromatography was carried out using Macherey-Nagel 60 230-400 mesh silica gel. All ^1H and ^{31}P NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer. NMR chemical shifts are reported in ppm downfield from an internal standard (TMS) or external standard (80% aqueous H_3PO_4). MALDI-TOF mass spectra were recorded on a Voyager-DE workstation (PE Biosystems). Matrixes used for preparing MALDI-TOF samples were following: a) 2,6-dihydroxyacetophenone, 20 mg/ml, and diammonium citrate, 40 mg/ml, in 50% aq. methanol, for all oligonucleotides and phosphoramidites and b) 2,5-dihydroxybenzoic acid, 10 mg/ml in methanol, for all other low molecular weight compounds. Elemental analyses were performed by Cambridge University Chemical Laboratory. Oligonucleotide analytical and semi-preparative HPLC was carried out on a Gilson HPLC chromatograph under Macintosh-powered Rainin Dynamax software, using a μ-Bondapak (Waters) or Phenomenex (ABI) RP-C18 column (0.5x25 cm) and dual wavelength (218 and 254 nm) detection.

Solid-Phase Oligonucleotide Synthesis. Assembly of oligonucleotides was carried out by the standard 2–cyanoethyl phosphoramidite method[31,34] on a LCAA-CPG support or a 3'-fluorescein CPG support, 3'-(6-FAM) CPG, obtained from Glen Research

via Cambio. All oligonucleotides were synthesised on a 1 μmol scale using an ABI 380B automatic DNA/RNA synthesizer according to manufacturer's instructions. All standard protected deoxyribonucleoside phosphoramidites and other synthesis reagents were obtained from Cruachem (Scotland). After the final 5'-dimethoxytrityl group removal, the cysteine phosphoramidite (**5-8**) (0.15 M solution in dry acetonitrile) was coupled to a support-bound oligonucleotide using an extended time (10 min) to ensure complete coupling. After the usual brief iodine oxidation, the support was manually flushed with 20% piperidine solution in DMF to remove the Fmoc-group for 10 min at room temperature, washed with 10 ml of DMF, 10 ml of acetonitrile and briefly air-dried. The support was then treated with 0.5 ml of 30% aqueous ammonia solution at room temperature for 2 h to cleave the succinate linker and the support washed with an additional 0.5 ml of concentrated ammonia solution. The combined filtrates were then transferred to a screw-capped polypropylene tube and heated at 55°C for 16 h to completely deprotect the oligonucleotide at nucleobase and phosphate moieties. After cooling and evaporation of most of the solution under a stream of nitrogen, 1 ml of deionised water was added and the solution was evaporated on a SpeedVac vacuum concentrator to dryness and redissolved in 200 μl deionized water. For the 3'-fluorescein oligonucleotide synthesis, the oligonucleotide product was precipitated with 2M lithium perchlorate in acetone (1.8 ml) at –20 °C for 10 min and after microcentrifugation and washing with acetone (2 x 2ml) the product was dissolved in water (200 μl). A quantity of the oligonucleotide was then assessed by checking absorbance at 260 nm on Perkin-Elmer Lambda 2 UV-Vis spectrophotometer. Purity of oligonucleotides was established by analytical reversed phase HPLC using a gradient of acetonitrile in 0.1M triethylammonium acetate buffer, pH 7.0. Correct masses were confirmed by MALDI-TOF mass spectroscopy (positive ion mode).

***N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (1).** To a slurry of *trans*-4-aminocyclohexanol hydrochloride (2 mmol, 303.3 mg), *N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) and 1-hydroxybenzotriazole (2 mmol, 270.3 mg) in anhydrous DMF (20 ml), was added triethylamine (3.1 mmol, 0.446 ml), and the resulting solution was stirred at room temperature for *ca.* 3 h, until TLC revealed the completion of the reaction. The mixture was then evaporated to dryness, the white residue transferred to sintered glass filter,

washed successively with small amount of DMF, EtOH and diethyl ether, and dried *in vacuo*. Yield of white powder 0.868 g (82%). TLC (A): R_f 0.39. ^{1}H-NMR (DMSO-d_6): δ 1.18 (m, 4H, CH_2), 1.27 (s, 9H, *tert*-butyl), 1.69 (m, 2H, CH_2), 1.77 (m, 2H, CH_2), 2.95 (m, 2H, CH_2S), 3.45 (m, 2H, CHO+CHN), 4.24 (m, 4H, Fmoc+α-CH), 4.50 (d, 1H, *J*=4.3 Hz, OH), 7.30 (t, 2H, *J*=7.4 Hz, fluorene), 7.41 (t, 2H, *J*=7.4 Hz, fluorene), 7.61 (d, 1H, *J*=8.5 Hz, NH), 7.72 (d, 2H, *J*=7.3 Hz, fluorene), 7.86 (m, 3H, fluorene+NH). MALDI-TOF MS: $[M+H]^+$ 528.9 (529.7 calc.), $[M+Na]^+$ 550.77 (551.72 calc.), $[M+K]^+$ 566.57 (567.69 calc.). Anal. Calcd. for $C_{28}H_{36}N_2O_4S_2$: C, 63.61; H, 6.86; N, 5.30. Found: C, 63.85; H, 6.84; N, 5.28.

***N*-α-Fmoc-*S*-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (2).** To a slurry of *trans*-4-aminocyclohexanol hydrochloride (2.1 mmol, 318.4 mg) and *N*-α-Fmoc-*S*-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in anhydrous DMF (25 ml) was added triethylamine (2.2 mmol, 0.307 ml), and resulting solution was stirred at room temperature for *ca.* 3 h, until TLC revealed completion of the reaction. The mixture was then evaporated to dryness, redissolved in ethyl acetate and washed successively with ice-cold 5% w/v citric acid solution, water, 5% sodium bicarbonate solution and brine, dried over anhydrous Na_2SO_4, and evaporated to a light brown foam. The residue was chromatographed on silica gel column eluted with 15-5% hexane in EtOAc containing 0.5% triethylamine. Appropriate fractions were pooled and evaporated to give 1.305 g (95%) of the title product as a white foam. TLC (A): R_f 0.49. ^{1}H-NMR (DMSO-d_6): δ 1.17 (m, 4H, CH_2), 1.65 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.29 (m, 2H, CH_2S), 3.36 (m, 2H, CHO+CHN), 4.01 (q, 1H, *J*=7.0 Hz, α-CH), 4.22 (m, 3H, Fmoc), 4.50 (d, *J*=4.3 Hz, OH), 7.28 (m, 17H, aromatics), 7.39 (t, 2H, *J*=7.4 Hz, fluorene), 7.57 (d, 1H, *J*=8.7 Hz, NH), 7.66 (d, 1H, *J*=7.68 Hz, NH), 7.72 (d, 2H, *J*=7.3 Hz, fluorene), 7.87 (d, 2H, *J*=7.5 Hz, fluorene). MALDI-TOF MS: $[M+Na]^+$ 704.1 (705.9 calc.), $[M+K]^+$ 720.05 (721.8 calc.). Anal. Calcd. for $C_{43}H_{42}N_2O_4S$: C, 75.63; H, 6.20; N, 4.10. Found: C, 75.33; H, 6.10; N, 4.11.

***N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine 4-hydroxypiperidide (3).** 4-Hydroxypiperidine (2.5 mmol, 253.1 mg) was added with stirring to a solution of *N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) in 20 ml of

anhydrous DMF. The reaction mixture was stirred for *ca.* 3 h at room temperature until TLC revealed completion of the reaction. The mixture was then evaporated to dryness, redissolved in EtOAc and washed successively with 5% w/v. citric acid solution (x2), H_2O, saturated sodium bicarbonate solution (x2) and brine, dried over anhydrous Na_2SO_4, and evaporated to a light yellow foam. The residue was chromatographed on a silica gel column eluted with 10-0% hexane in EtOAc, appropriate fractions were pooled and evaporated to give 0.9831 g (95%) of the title product as a white foam. TLC (A): R_f 0.36. ^{1}H-NMR (DMSO-d_6): δ 1.28 (s, 11H, *tert*-butyl+CH_2), 1.69 (m, 2H, CH_2), 2.91 (m, 2H, CH_2S), 3.11 (m, 2H, CH_2N), 3.68 (m, 2H, CH_2N), 3.87 (m, 1H, CHO), 4.20 (t, 1H, *J*=6.6 Hz, 9-fluorene), 4.28 (d, 2H, *J*=6.5 Hz, Fmoc CH_2), 4.72 (m, 1H, α-CH), 7.31 (t, 2H, *J*=7.3 Hz, fluorene), 7.41 (t, 2H, *J*=7.2 Hz, fluorene), 7.71 (d, 2H, *J*=7.7 Hz, fluorene), 7.88 (d, 2H, *J*=7.5 Hz, fluorene). MALDI-TOF MS: $[M+H]^+$ 514.8 (515.7 calc.), $[M+Na]^+$ 536.3 (537.7 calc.), $[M+K]^+$ 552.1 (553.7 calc.).

***N*-α-Fmoc-*S*-trityl-L-cysteine 4-hydroxypiperidide (4).** 4-Hydroxypiperidine (2.3 mmol, 232.6 mg) was added with stirring to the solution of *N*-α-Fmoc-*S*-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in 25 ml of anhydrous MeCN, followed by NEt_3 (0.5 mmol, 0.07 ml). Reaction mixture was stirred at room temperature for 4 h, until TLC revealed the completion of the reaction. The mixture was then evaporated to dryness, redissolved in EtOAc and washed successively with ice-cold 5% wt. citric acid (x2), H_2O, saturated $NaHCO_3$ solution (x2), and brine, dried over anhydrous Na_2SO_4, and evaporated to a light brown foam. The residue was chromatographed on silica gel column eluted by 10-25% of MeCN in $CHCl_3$, appropriate fractions were pooled and evaporated to give 1.3126 g (98%) of the title product as a white foam. TLC (A): R_f 0.41. ^{1}H-NMR (DMSO-d_6): δ 1.15 (m, 2H, CH_2), 1.53 (m, 2H, CH_2), 2.49 (m, 2H, CH_2S), 2.87 (m, 2H, CH_2N), 3.24 (m, 2H, CH_2N), 3.60 (m, 1H, CHO), 3.82 (m, 1H, α-CH), 4.24 (m, 3H, Fmoc), 4.71 (d, 1H, *J*=4.0 Hz, OH), 7.31 (m, 19H, aromatics), 7.70 (d, 2H, *J*=7.4 Hz, fluorene), 7.83 (t, 1H, NH), 7.87 (d, 2H, *J*=7.5 Hz, fluorene). MALDI-TOF MS: $[M+Na]^+$ 690.6 (691.8 calc.), $[M+K]^+$ 706.5 (707.8 calc.).

4-*trans*-*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinylamidocyclohexyl 2-cyanoethyl *N*,*N*-diisopropyl phosphoramidite (5). To a chilled (ice bath) solution of 4-*trans*-*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinylamidocyclohexanol (0.834 g, 1.577 mmol) in 15 ml

of anhydrous dichloromethane containing 3 eq (0.785 ml) of DIEA, 2-cyanoethoxy-*N,N*-diisopropylaminochlorophosphine (1.5 eq, 0.529 ml) was added dropwise *via* syringe ander nitrogen. After 1 h of stirring cold, the mixture was allowed to warm up gradually, and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of MeOH, evaporated to dryness, and the residue was taken up in EtOAc, washed with saturated $NaHCO_3$ solution (x2) and brine, dried over anhydrous Na_2SO_4 and evaporated to a small volume. The residue was chromatographed on a silica gel column eluted with 15-40% of EtOAc in hexane containing 2% triethylamine, appropriate fractions pooled, evaporated to dryness and co-evaporated with dry CH_2Cl_2 to give a white foam. Yield of title product 0.757 g (66%). TLC (B): R_f 0.58. ^{1}H-NMR (CD_3CN): δ 1.16 (d, 6H, *J*=0.9 Hz, *iso*-propyl CH_3), 1.18 (d, 6H, *J*=1.0 Hz, *iso*-propyl CH_3), 1.27 (m, 2H, CH_2), 1.32 (s, 9H, *tert*-butyl), 1.45 (m, 2H, CH_2), 1.87 (m, 2H, CH_2), 2.01 (m, 2H, CH_2), 2.64 (t, 2H, *J*=5.9 Hz, CH_2CN), 3.03 (m, 2H, CH_2S), 3.69 (m, 6H, CHO+CHN+CH_2O+*iso*-propyl CHN), 4.32 (m, 4H, Fmoc+α-CH), 6.07 (d, 1H, *J*=8.6 Hz, NH), 6.59 (d, 1H, *J*=7.8 Hz, NH), 7.34 (t, 2H, *J*=7.5 Hz, fluorene), 7.43 (t, 2H, *J*=7.4 Hz, fluorene), 7.68 (d, 2H, *J*=7.3 Hz, fluorene), 7.85 (d, 2H, *J*=7.5 Hz, fluorene). ^{31}P-NMR (CD_3CN) δ 146.51 ppm. MALDI-TOF MS: $[M+H]^+$ 729.4.0 (730.0 calc.).

4-*trans*-*N*-α-Fmoc-*S*-trityl-L-cysteinylamidocyclohexyl 2-cyanoethyl *N,N*-diisopropyl phosphoramidite (6). To a solution of *N*-α-Fmoc-*S*-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (0.344 mmol, 0.235 g) in 10 ml of anhydrous CH_2Cl_2 containing 75 mg (1.5 eq) of diisopropylammonium tetrazolide, 2-cyanoethoxy-*N,N,N',N'*-tetraisopropyl phosphordiamidite (1.15 eq, 0.126 ml) was added, and the mixture was stirred for 6 h at room temperature, until TLC (B) revealed complete reaction. Dichloromethane was then removed by evaporation, the residue was taken up in EtOAc, washed with saturated $NaHCO_3$ solution (x2) and brine, dried over anhydrous Na_2SO_4 and evaporated to a small volume. The residue was chromatographed on silica gel column eluted with 15-40% EtOAc in hexane containing 2% NEt_3, appropriate fractions were pooled and evaporated to dryness. TLC (B): R_f 0.76.Yield of a title product 1.305 g (95%). ^{1}H-NMR (DMSO-d_6): δ 1.11 (d, 6H, *J*=1.6 Hz, *iso*-propyl CH_3), 1.13 (d, 6H, *J*=1.4 Hz, *iso*-propyl CH_3), 1.19 (m, 2H, CH_2), 1.35 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 1.86 (m, 2H, CH_2), 2.28 (m, 2H, CH_2S), 2.76 (t, 2H, *J*=5.8 Hz, CH_2CN), 3.57

(m, 6H, CHO+CHN+CH_2O+*iso*-propyl CHN), 4.02 (q, 1H, *J*=7.1 Hz, α-CH), 4.22 (m, 3H, Fmoc), 7.29 (m, 17H, aromatics), 7.39 (t, 2H, *J*=7.5 Hz, fluorene), 7.59 (d, 1H, *J*=8.6 Hz, NH), 7.72 (m, 3H, fluorene+NH), 7.88 (d, 2H, *J*=7.5 Hz, fluorene). ^{31}P-NMR (CD_3CN) δ 146.50 ppm. MALDI-TOF MS: $[M+H]^+$ 922.0 (922.2 calc.).

4-*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinylpiperidyl 2-cyanoethyl *N,N*-diisopropyl phosphoramidite (7). To a solution of *N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine 4-hydroxypiperidide (1.536 mmol, 0.790 g) in 15 ml of anhydrous CH_2Cl_2 containing 0.329 g (1.920 mmol) of diisopropylammonium tetrazolide, 2-cyanoethoxy-*N,N,N',N'*-tetraisopropyl phosphordiamidite (1.690 mmol, 0.537 ml) was added under N_2 atmosphere, and the mixture was stirred for 3 h at room temperature, until TLC (B) revealed complete reaction. The solvent was then removed by evaporation, the residue was taken up in EtOAc, washed with saturated $NaHCO_3$ solution (x2) and brine, dried over anhydrous Na_2SO_4, filtered through a cotton plug, evaporated to a white foam, re-evaporated several times with CH_2Cl_2, dried *in vacuo* and dissolved in small volume of 10% EtOAc in hexane. The solution was chromatographed on a silica gel column eluted with 10-40% EtOAc in hexane containing 2% triethylamine, appropriate fractions were pooled, evaporated to dryness and rinsed with hexane. The remaining waxy solid was dissolved in dry CH_2Cl_2, and re-evaporated twice with dry CH_2Cl_2 to give a white foam. Yield of title product 0.455 g (42%). TLC (B): R_f 0.78. ^{1}H NMR (CD_3CN): δ 1.17 (m, 12H, *iso*-propyl CH_3), 1.33 (s, 9H, *tert*-butyl), 1.61 (m, 2H, CH_2), 1.82 (m, 2H, CH_2), 2.64 (t, 2H, *J*=5.9 Hz, CH_2CN), 2.98 (m, 2H, CH_2S), 3.44 (m, 2H, CH_2O), 3.68 (m, 6H, CH_2N+*iso*-propyl CHN), 4.09 (m, 1H, CHO), 4.24 (t, 1H, *J*=6.7 Hz, 9-fluorene), 4.36 (d, 2H, *J*=6.0 Hz, Fmoc CH_2), 4.87 (q, 1H, *J*=6.3 Hz, α-CH), 6.15 (m, 1H, NH), 7.34 (t, 2H, *J*=7.3 Hz, fluorene), 7.43 (t, 2H, *J*=7.2 Hz, fluorene), 7.67 (d, 2H, *J*=7.3 Hz, fluorene), 7.84 (d, 2H, *J*=7.4 Hz, fluorene). ^{31}P NMR (CD_3CN): δ 147.10 (61%), 147.03 (19%), 146.94 (20%) (mixture of rotamers). MALDI-TOF MS: $[M+H]^+$ 715.7 (715.9 calc.), $[M+Na]^+$ 737.0 (737.9 calc.), $[M+K]^+$ 753.4 (754.0 calc.).

4-*N*-α-Fmoc-*S*-trityl-L-cysteinylpiperidyl 2-cyanoethyl *N,N*-diisopropyl phosphoramidite (8). To a chilled (ice bath) solution of *N*-α-Fmoc-*S*-trityl-L-cysteine 4-hydroxypiperidide (0.668 g, 1 mmol) in 10 ml of anhydrous CH_2Cl_2 containing 3 mmol (0.514 ml) of DIEA, 2-cyanoethoxy-*N,N*-diisopropylaminochlorophosphine (1.3 mmol,

0.29 ml) was added dropwise via syringe under N_2. After 1 h of stirring cold, the mixture was allowed to warm up gradually, and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of MeOH, evaporated to dryness, the residue was taken up in EtOAc, washed with saturated $NaHCO_3$ solution (x2) and brine, dried over anhydrous Na_2SO_4 and evaporated to a small volume. This was chromatographed on a silica gel column eluted with 15-30% EtOAc in hexane containing 2% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of title product 0.695 g (80%). TLC (C): R_f 0.39. ^{1}H NMR (CD_3CN): δ 1.20 (m, 12H, *iso*-propyl CH_3), 1.48 (m, 2H, CH_2), 1.69 (m, 2H, CH_2), 2.46 (d, 2H, *J*=6.5 Hz, CH_2S), 2.63 (m, 2H, CH_2CN), 3.32 (m, 2H, CH_2O), 3.87 (m, 6H, CH_2N+*iso*-propyl CHN), 4.00 (m, 1H, CHO), 4.22 (t, 1H, *J*=6.8 Hz, 9-fluorene), 4.32 (m, 2H, Fmoc CH_2), 4.42 (m, 1H, α-CH), 6.03 (m, 1H, NH), 7.32 (m, 21H, aromatics), 7.66 (d, 2H, *J*=6.6 Hz, fluorene), 7.83 (d, 2H, *J*=7.5 Hz, fluorene). ^{31}P NMR (CD_3CN): δ 147.07 (67%), 146.96 (18%), 146.91 (15%) (mixture of rotamers). MALDI-TOF MS: $[M+H]^+$ 892.2 (892.1 calc.), $[M+Na]^+$ 908.2 (908.2 calc.).

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REFERENCES

1. Crooke, S. T.; Lebleu, B. (Eds.) *Antisense Research and Applications*, **1993,** CRC Press: Boca Raton, USA.
2. Chadwick, D. J.; Cardew, G. *Oligonucleotides as therapeutic agents*, **1997**, John Wiley, Chichester, UK.
3. Lewis, J. G.; Lin, K.-Y.; Kothavale, A.; Flanagan, W. M.; Matteucci, M. D.; DePrince, R. B.; Mook, Jr., R. A.; Hendren, R. W.; Wagner, R. W. *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 3176-3181.
4. Morris, M. C.; Vidal, P.; Chaloin, L.; Heitz, F.; Divita, G. *Nucleic Acids Res.* **1997**, *25*, 2730-2736.

5. Pichon, C.; Freulon, I.; Midoux, P.; Mayer, R.; Monsigny, M.; Roche, A.-C. *Antisense Nucleic Acid Drug Dev.*, **1997**, *7*, 335-343.
6. Wyman, T.B.; Nicol, F.; Zelphati, O.; Scario, P.V.; Plank, C.; Szoka, F.C. *Biochemistry*, **1997**, *36*, 3008-3017.
7. Midoux, P.; Kichler, A.; Boutin, V.; Maurizot, J.-C.; Monsigny, M., M. *Bioconjug. Chem.* **1998**, *9*, 260-267.
8. Bongartz, J. -P.; Aubertin, A. -M.; Milhaud, P. G.; Lebleu, B. *Nucleic Acids Res.* **1994**, *22*, 4681-4688.
9. Pichon, C.; Arar, K.; Stewart, A. J.; Dodon, M. D.; Gazzolo, L.; Courtoy, P. J.; Mayer, R.; Monsigny, M; Roche, A. -C. *Mol. Pharmacol.*, **1997**, *51*, 431.
10. Bachmann, A. S.; Surovoy, A.; Jung, G.; Moelling, K. *J. Mol. Med.*, **1988**, *76* 126.
11. Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Auriola, S.; Jaaskelainen, I.; Ronkko, S.; Honkakoski, P.; Urtti, A.; Lönnberg, H.; Azhayev, A. *Bioconjug. Chem.* **1999**, *10*, 598-606.
12. Zanta, M.-A.; Belguise-Valladier, P.; Behr, J.-P. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 91-96.
13. Allinquant, B.; Hantraye, P.; Mailleux, P.; Moya, K.; Bouillot, C.; Prochiantz, A. J. *Cell Biol.*, **1995**, *128*, 919-927.
14. Prochiantz, A. *Current Opin. Neurobiol.*, **1996**, *6*, 629-634.
15. Derossi, D.; Chassaing, G.; Prochiantz, A. *Trends in Cell Biol.*, **1998**, *8*, 84-87.
16. Chaloin, L.; Vidal, P.; Lory, P.; Méry, J.; Lautredou, N.; Divita, G.; Heitz, F. *Biochem. Biophys. Res. Comm.*, **1998**, *243*, 601-608.
17. Haralambidis, J.; Duncan, L.; Angus, K; Tregear, G. W. *Tetrahedron Lett.*, **1987**, *28*, 5199-5202.
18. De La Torre, B. G.; Avinó, A.; Tarrason, G.; Piulats, J.; Albericio, F.; Eritja, R. *Tetrahedron Lett.*, **1994**, *35*, 2733-2736.
19. Bergmann, F.; Bannwarth, W. *Tetrahedron Lett.*, **1995**, *36*, 1839-1842.
20. Basu, S.; Wickstrom, E., *Tetrahedron Lett.*, **1995**, *36*, 4943-4946.
21. De Napoli, L.; Messere, A.; Montesarchio, D.; Piccialli, G.; Benedetti, E.; Bucci, E.; Rossi, F. *Bioorg. Med. Chem.*, **1999**, *7*, 395-400.
22. Antopolsky, M.; Azhayev, A. *Helv. Chim Acta*, **1999**, *82*, 2130-2140.
23. Arar, K.; Aubertin, J. -M.; Roche, A. -C.; Monsigny, M. M.; Mayer, R. *Bioconjug. Chem.* **1995**, *6*, 573-577.

24. Soukchareun, S.; Treager, G. W.; Haralambidis, J. *Bioconjug. Chem.* **1995**, *6*, 43-53.

25. Vivès, E.; Lebleu, B. *Tetrahedron Letters*, **1997**, *38*, 1183-1186.

26. Peyrottes, S.; Mestre, B.; Burlina, F.; Gait, M. J. *Tetrahedron*, **1998**, *54*, 12513-12522.

27. Eritja, R.; Pons, A.; Escarceller, M.; Giralt, E.; Albericio, F., *Tetrahedron.* **1991**, *47*, 4113-4120.

28. Tung, C. -H.; Rudolph, M. J.; Stein, S. *Bioconjug. Chem.*, **1991**, *2*, 464-465.

29. Harrison, J. G.; Balasubramanian, S. *Nucleic Acids Res.*, **1998**, *26*, 3136-3145.

30. Soukchareun, S.; Haralambidis, J.; Tregear, G. *Bioconjug. Chem.* **1998**, *9*, 466-475.

31. Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucl. Acids Res.*, **1984**, *12*, 4539-4557.

32. Connolly, B. A.; Rider, P. *Nucl. Acids Res.* **1985**, *13*, 4485-4502.

33. Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. M. *J. Org. Chem.*, **1991**, *56*, 2648-2650.

34. Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-1862.

35. Stetsenko, D. A.; Gait, M. J. *J. Org. Chem.*, **2000**, in press.

36. Muir, T. W.; Dawson, P. E.; Kent, S. B. H. *Methods Enzymol.*, **1997**, *289*, 266-298.

37. Wilken, J;, Kent, S. B. H. *Current Opin. Biotechnol.*, **1998**, *9*, 412-426.

38. Dawson, P. E.; Griffin, J. H.; Hackeng, T. M. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 10068-10073.

39. Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Amer. Chem. Soc.*, **1999**, *121*, 11369-11374.

40. Shin, Y.; Winans, K.; Backes, B.; Kent, S. B. H.; Ellmann, J. A.; Bertozzi, C. R. *J. Amer. Chem. Soc.*, **1999**, *121*, 11684-11689.