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Solid-phase synthesis of H-Phe-Tyr-(pATAT)-NH_2: a nucleopeptide fragment from the nucleoprotein of bacteriophage ϕX174

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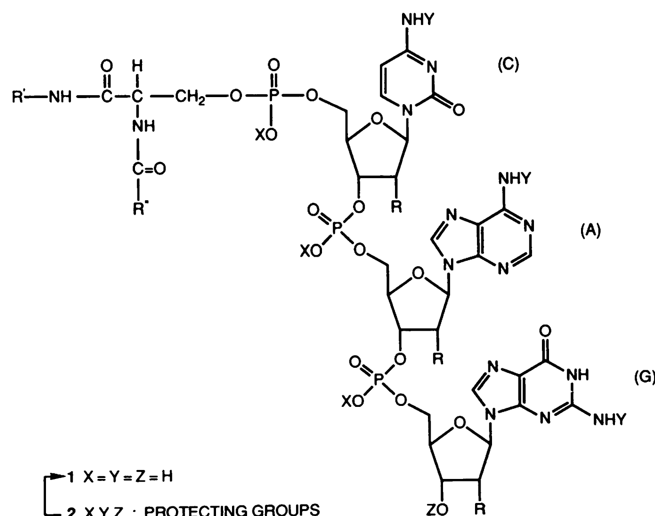
ABSTRACT

The preparation of the nucleopeptide H-Phe-Tyr-(pATAT)-NH_2 could be realized *via* a solid phase phosphitetriester approach and by using the protected protecting group 2-(*tert*-butyldiphenylsilyloxymethyl)-benzoyl for the masking of the N^6-amino function of deoxyadenosine. The latter protecting group can be removed under mild conditions with fluoride ion.

INTRODUCTION

Nucleoproteins are naturally occurring biopolymers[1-5] (*e.g.* **1**) in which the 5'-terminal hydroxyl group of nucleic acids [DNA (*e.g.* **1**, R=H) or RNA (*e.g.* **1**, R=OH)] is covalently linked *via* a phosphodiester bond with the respective hydroxyl groups of the L-amino acids serine (*e.g.*, **1**), threonine and (or) tyrosine in proteins. Thus far, synthetic efforts directed towards the preparation of nucleopeptide fragments (*i.e.*, **1**), containing the rather base-labile nucleotide-(P-O)-serine bond, clearly indicated[6-8] that a general and reliable synthetic route towards this type of biopolymers was *inter alia* severely hampered[9] by the lack of suitably protecting groups (*i.e*, Y in **2**) for the exocyclic amino functions of the nucleobases adenine, cytosine and guanine.



Recently we reported[10] that the exocyclic amino protecting group 2-(*tert*-butyldiphe-nylsilyloxymethyl)-benzoyl (SiOMB) could be deblocked under virtually neutral conditions with fluoride ion.

We now report that the nucleopeptide H-Phe-Tyr(pATAT)-NH_2 (*i.e.*, compound **18**), which is a fragment of the nucleoprotein formed in the initial stage of the rolling circle replication of double stranded circular DNA of bacteriophage ϕX 174 mediated by gene A protein[11-12], can be prepared *via* a solid-phase approach using the SiOMB group for the protection of the exocyclic amino functions of the adenine moieties.

RESULTS AND DISCUSSION

The preparation of the required building unit **8** of deoxyadenosine is outlined in Scheme 1 and commences with the synthesis of the silyl-protected acid chloride SiOMB-Cl (**4**). Thus, silylation of 2-bromo-benzyl alcohol (**3**) with *tert*-butyldiphenylsilyl chloride and subsequent treatment of 2-(*tert*-butyldiphenyl-silyloxymethyl)-bromobenzene thus obtained with magnesium, gave, after carboxylation of the Grignard derivative with gaseous carbon dioxide, the corresponding benzoic acid derivative. The latter compound was easily converted with oxalyl chloride to furnish SiOMB-Cl (**4**) in 63% overall yield.

The preparation of key compound N^6-SiOMB-deoxyadenosine **6a** was now accomplished as follows. Transient protection of **5** with trimethylsilyl groups using hexamethyldisilazane and trimethylsilyl chloride, according to McGee *et al.*[13], followed by treatment with **4** resulted, as gauged by TLC-analysis, in the formation of **6a** and a small quantity of the corresponding N^6-di-SiOMB product. Fortunately, the latter compound could be converted quantitatively into **6a** by short treatment with sodium methoxide in methanol. Tritylation of **6a** with 4,4'-dimethoxytrityl chloride gave, after purification by silica gel chromatography, homogeneous **6b** in 75% overall yield. Phosphitylation of **6b** with 2-cyanoethyl N,N-diisopropylaminochlorophosphite (**7**), following the procedure of Sinha *et al.*[14], gave, after purification, the homogeneous phosphoramidite **8** in 90% yield.

The immobilized and partially protected tetramer **14** (5'-hydroxyl free) was now assembled, using an automated Gene

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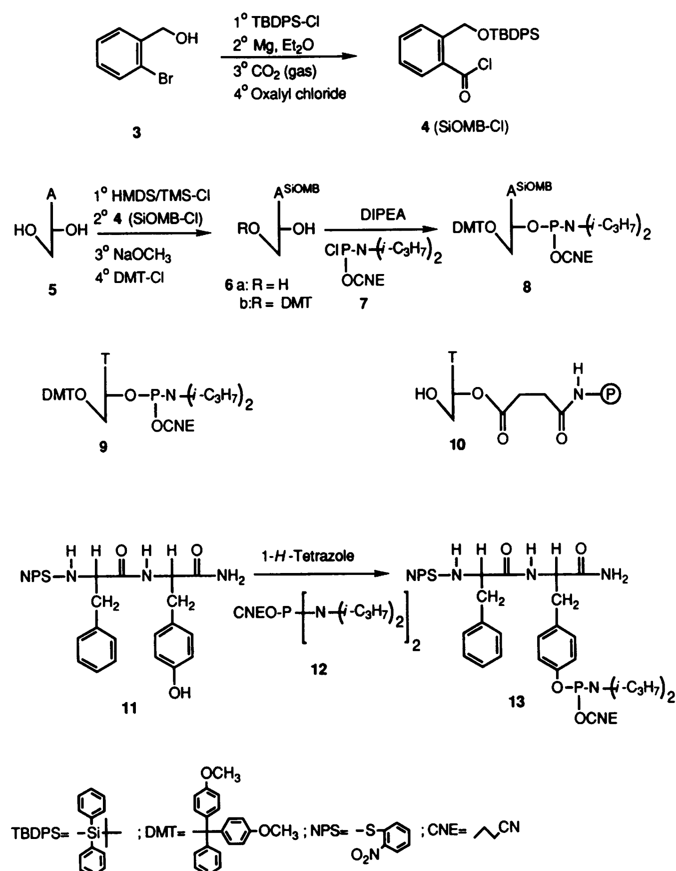


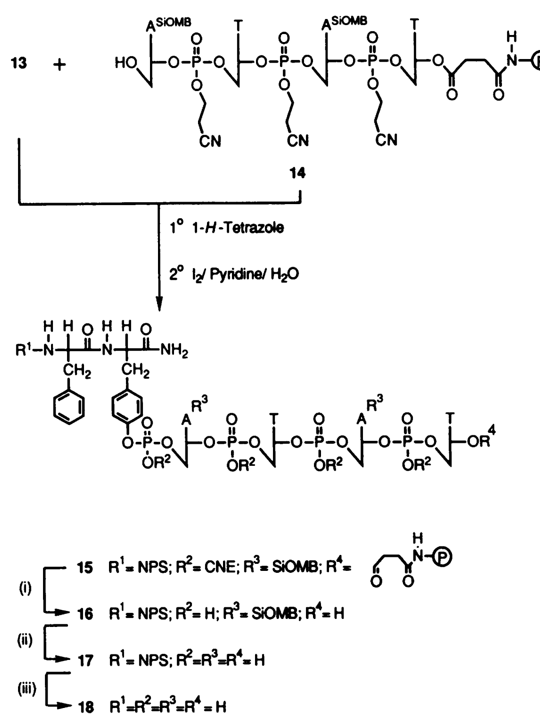
Assembler (Pharmacia), by stepwise elongation (see Table 1) of immobilized thymidine **10**, linked by a 3'-O-succinyl bond to monobeads (Pharmacia), *via* 1-*H*-tetrazole assisted coupling of the respective phosphoramidites **8** and **9**, followed by oxidation of the resulting phosphitetriesters and subsequent acidolysis of the individual DMT-groups. The efficacy of each elongation cycle was, as monitored spectro-photometrically by the released DMT-cation, higher than 97%.

At this stage, the phenolic hydroxyl group of the dipeptide **11** (Scheme 1) was phosphitylated with 2-cyanoethyl-*bis*(N,N-diisopropylamino)phosphite[15] (**12**), in the presence of 1-*H*-tetrazole, to give, after silica gel chromatography, the phosphoramidite **13** in 74% yield. In this respect, it is of interest to note that phosphitylation of dipeptide **11** with reagent **7** was not selective. Thus monitoring of the reaction by ^{31}P NMR spectroscopy showed the appearance of additional phosphorus-reasonances (*i.e.* δ 117.4 and 117.8 ppm), which may be due to the unwanted phosphitylation of the NPS-protected amino function in **11**. 1-*H*-Tetrazole assisted coupling (Scheme 2) of **13** with the immobilized tetramer **14** was executed under the same conditions as described for step 2 in Table I. Oxidation (step 3 in Table I) of the intermediate phosphitetriester led to the fully protected and immobilized nucleopeptide **15**. The formation of **15** was corroborated indirectly by the outcome of the following three-step deblocking procedure (see further Figure 1). Thus, short ammonolysis of **15** effected the release from the solid support and the elimination of the cyanoethyl (R^2) groups. Analysis of crude product **16** thus obtained by reversed phase chromatography (see Figure 1A) revealed the presence of mainly

Table 1. Chemical steps involved in each elongation cycle.

Step	*Manipulation*	*Solvents and reagents*[a]	*Time (min)*
1	Detritylation	2% Trichloroacetic acid in 1,2-dichloroethane	2.5
2	Coupling	**8**[b] or **9**[b], 1-*H*-tetrazole[c], MeCN	3.0
3	Oxidation	0.02 M I_2 in MeCN/2,4,6-collidine/ H_2O (10:1:5)	1.0
4	Capping	0.25 M 4-dimethylaminopyridine in Ac_2O/2,4,6-collidine/MeCN (1:1:8)	1.2

[a]Reactions were performed on 200 mg (10μmol) of resin **10**. [b]0.1M **8** or **9** (0.5 ml) in MeCN. [c]0.5 M 1-*H*-tetrazole (0.85 ml) in MeCN.



one product. Removal of the SiOMB (R^3) groups from **16** with fluoride ion gave, as evidenced by reversed phase chromatography (see Fig. 1B) predominantly **17**. Finally, removal of the NPS (R^1) group from crude **17** with 2-mercaptopyridine (see Fig. 1C) yielded, after successive purification by gelfiltration and anion-exchange chromatography, homogeneous **18** (Na^+-salt; 1.8 mg). The analytical (FPLC) and spectroscopic (^{1}H- and ^{31}P NMR) data of **18** were in complete accord with the proposed structure and, further, in excellent agreement with those of the same nucleopeptide[7] prepared earlier in solution *via* a phosphatetriester approach.

In conclusion, we believe that the solid phase approach described in this paper may be of great value for the synthesis of biologically important nucleopeptides, the tyrosine moiety of which may be either linked to the 5'-end of DNA or RNA by a phosphodiester bond.

At present we are studying in detail whether the above described methodology can be adapted for the preparation of nucleopeptides containing the intrinsically base labile nucleotide-(P-O)-serine linkage.

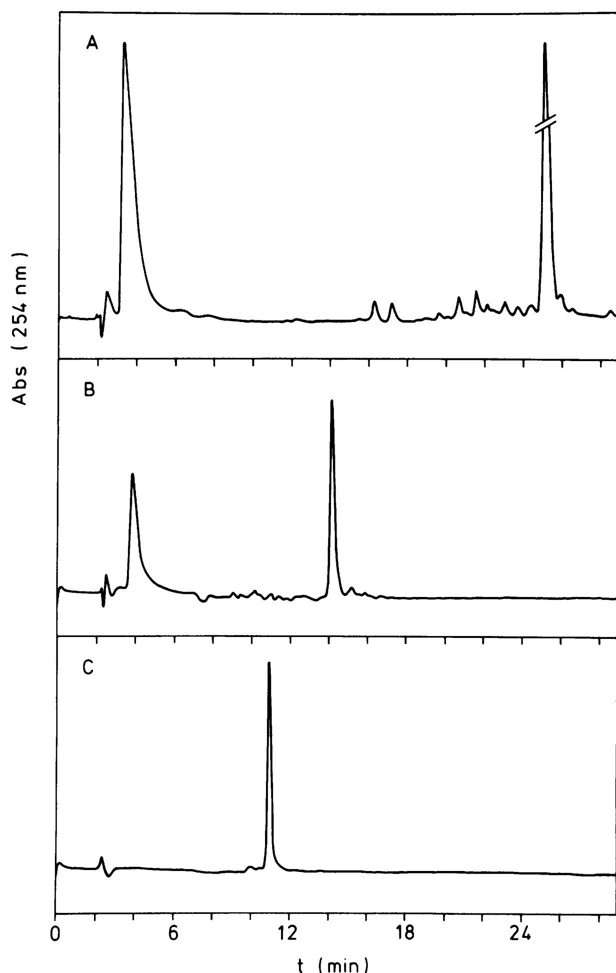


Figure 1: FPLC (reversed phase) profiles of the crude products **16** (A); **17** (B) and **18** (C).

EXPERIMENTAL

Pyridine, tetrahydrofuran *N,N*-diisopropylethylamine and dioxane were dried by refluxing with calcium hydride (5 g/l), distilled and stored over molecular sieves (4Å). Tetrahydrofuran and dioxane were redistilled prior to use from lithium aluminium hydride (5 g/l). Toluene and xylene were distilled from phosphorus pentoxide and stored over sodium wire. Triethylammonium bicarbonate buffer (2 M) was prepared by passing a stream of carbon dioxide through a cooled (0°C) solution of triethylamine in water until a neutral solution was obtained. 2-Cyanoethyl-*N,N*-diisopropylaminochlorophosphite and 2-cyanoethyl *bis*-(*N,N*-diisopropylamino)phosphite were prepared according to the literature. 2-Bromobenzyl alcohol, *tert*-butyldiphenylsilyl chloride and hexamethyldisilazane were purchased from Janssen Chimica. 1-*H*-tetrazole, 5'-*O*-dimethoxytritylthymidilyl-3'-*O*-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (**9**) and functionalized monobeads were purchased from Pharmacia. TLC analysis was performed on Schleicher and Schüll Fertigfolien F1500 LS254 with solvents systems: A (hexane/ethyl acetate, 90/10, v/v); B (dichloromethane/methanol, 90/10, v/v); C (dichloromethane/methanol, 95/5, v/v); D (hexane/ethyl acetate/triethylamine, 30/67.5/2.5, v/v/v); E (hexane/ethyl acetate/triethylamine, 10/89/1, v/v/v). Short column chromatography was performed on Kieselgel 60, 230–400 mesh (Merck). ^{1}H NMR spectra were measured at 200 or 300 MHz, using a JEOL JNM-FX 200 spectrometer or a Bruker WM-300 spectrometer, equipped with an ASPECT-2000 computer, operating in the Fourier Transform mode, respectively. Tetramethylsilane was used as internal reference for samples in deutero-chloroform. Chemical shifts are given in ppm (δ) relative to tetramethylsilane. ^{31}P NMR spectra were measured at 80.7 MHz, proton-noise decoupled, using a JEOL JNM-FX 200 spectrometer. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard. ^{13}C NMR spectra were measured at 50.3 MHz, using a JEOL JNM-FX 200 spectrometer. FPLC analysis was carried out on a Pharmacia LCC-500 liquid chromatographe using a MonoQ HR 5/5 column (anion exchange) or a ProRPC HR 5/10 column (reversed phase). Gradient elution was performed at 20°C by building up a gradient starting with buffer A (0.01 M sodium hydroxide (anion exchange); 0.1 M triethylammonium acetate in water pH=7 (reversed phase) and applying buffer B (0.01 M sodium hydroxide and 1.2 M sodium chloride (anion exchange); 0.1 M triethylammonium acetate in water/acetonitrile, 1/9, v/v, pH=7 (reversed phase) at a flow rate of 2.0 ml/min (anion exchange) and 0.75 ml/min (reversed phase). Gelfiltration was carried out on a Hiload Sephacryl S400 HR 26/60 column, by elution with 0.15 M triethylammonium hydrogen carbonate buffer at a flow rate of 1.5 ml/min. Solid phase synthesis was performed on a Gene Assembler (Pharmacia). The synthesis was conducted on a 10 μmol scale using derivatized monobeads. Solvents were HPLC-grade and dried over molecular sieves (3Å, Fluka) before use.

2-(*tert*-butyldiphenylsilyloxymethyl)-bromobenzene

2-Bromobenzyl alcohol (9.4 g, 50 mmol) was evaporated with anhydroTus pyridine (50 ml) and dissolved in pyridine (100 ml), and *tert*-butyldiphenylsilyl chloride (14.3 ml, 55 mmol) was added at 0°C. After stirring for 5 h at 20°C, the reaction mixture was concentrated. The residue was taken up in diethyl ether (500 ml), washed with water (100 ml), aqueous sodium hydrogen carbonate (10/90, w/v, 100 ml) and water (100 ml). The organic layer was dried over magnesium sulfate and the solvent was removed *in vacuo*. The product was purified by column chromatography (150 g, hexane/ethyl acetate, 100/0 to 85/15, v/v) to afford a colourless oil (19.1 g, 90% yield).

TLC R_f=0.51 (system A); ^{1}H NMR ($CDCl_3$) δ 1.12 (s, 9H, $C(CH_3)_3$), 4.79 (s, 2H, CH_2), 7.10–7.76 (m, 14H, aromatic H); ^{13}C NMR ($CDCl_3$) δ 19.4 ($\mathit{C}(CH_3)_3$), 26.9 ($C(\mathit{C}H_3)_3$), 65.3 (CH_2), 121.2 (CBr), 127.3–135.5 (CH, aromatic), 133.2 (CSi, phenyl), 140.0 ($\mathit{C}CH_2O$).

2-(*tert*-butyldiphenylsilyloxymethyl)-benzoic acid

2-(*Tert*-butyldiphenylsilyloxymethyl)-bromobenzene (19.1 g, 45 mmol) was coevaporated with anhydrous toluene and dissolved in dry tetrahydrofuran (40 ml). The solution was added dropwise to magnesium turnings (1.6 g, 67.5 mmol) under a nitrogen atmosphere over a period of 1 h. After stirring for another hour, a powerful stream of carbon dioxide was passed through the solution (1 h). The reaction mixture was cooled to 0°C and quenched with potassium hydrogen sulfate (200 ml, 1.0 M). After addition of diethyl ether (500 ml), the organic layer was separated, washed with potassium hydrogen sulfate (100 ml, 1.0 M) and water (100 ml). The organic layer was dried over magnesium sulfate and concentrated *in vacuo*. The crude product was crystallized from hexane (12.3 g, 70% yield).

Mp 142 °C; TLC R_f= 0.10 (system A); IR (KBr): 1685 cm^{-1}

(C=O); Mass spectrum m/e 391 (MH^+); ^1H NMR ($CDCl_3/CD_3OD$) δ 1.13 (s, 9H, $C(CH_3)_3$), 5.04 (s, 2H, CH_2), 7.19−8.27 (m, 14H, aromatic H); ^{13}C NMR ($CDCl_3$) δ 19.4 ($C(CH_3)_3$), 26.9 ($C(CH_3)_3$), 64.2 (CH_2), 126.1 (CC=O), 126.1−135.5 (CH, aromatic) 133.3 (CSi, phenyl), 144.3 (CCH_2), 172.6 (C=O).

2-(*tert*-butyldiphenylsilyloxymethyl)-benzoyl chloride (4)

2-(*Tert*-butyldiphenylsilyloxymethyl)-benzoic acid (11.7 g, 30 mmol) and oxalyl chloride (6.5 ml, 75 mmol) in dry toluene (75 ml) were heated for one hour at 50°C. The reaction mixture was concentrated *in vacuo* and coevaporated with toluene (2×50 ml). A stock solution (0.5 M of **4**) in dioxane could be stored for several weeks at −20°C.

5′-*O*-(4,4′-Dimethoxytrityl) 6-*N*-2(*tert*-butyldiphenylsilyloxymethyl)-benzoyl deoxyadenosine (6b)

Deoxyadenosine (1.26 g, 5.0 mmol) was suspended in dry acetonitrile, hexamethyl disilazane (8.4 ml, 80 mmol) and trimethylsilyl chloride (0.1 ml, 0.8 mmol) were added. After stirring for 1 h at 20°C, the precipitate was removed by filtration. The filtrate was concentrated *in vacuo* and coevaporated with xylene (15 ml). Dry pyridine (50 ml) and 2-(*tert*-butyldiphenylsilyloxymethyl)-benzoyl chloride (22.5 ml, 0.5 M) were added and the reaction mixture was stirred for 2 h at 20°C. The reaction was quenched with methanol (0.5 ml) and concentrated *in vacuo*. The reaction mixture was taken up in ethyl acetate (100 ml), washed with aqueous sodium chloride (2×25 ml, saturated), dried over magnesium sulfate and concentrated *in vacuo* to give the completely blocked nucleoside as a foam. The latter was dissolved in dioxane (25 ml) and sodium methoxide (30.0 ml, 1.0 M) was added to the cooled (0°) solution. After stirring for 5 min, the reaction was neutralized by addition of acetic acid. The mixture was diluted with dichloromethane (200 ml) and washed with aqueous sodium chloride (2×50 ml, saturated). The organic layer was dried over magnesium sulfate and concentrated *in vacuo*. The residu was coevaporated with dry pyridine (50 ml), dissolved in dry pyridine (50 ml) and 4,4′-dimethoxytrityl chloride (1.78 g, 5.25 mmol) was added. After stirring for 1 h, methanol (1.0 ml) was added, and the solvents were evaporated. The residue was taken up in dichloromethane (200 ml), washed with aqueous sodium hydrogen carbonate (10/90, w/v, 50 ml) and water (50 ml). The organic layer was dried over magnesium sulfate and the solvent was removed *in vacuo*. The product was purified by column chromatography (50 g, dichloromethane/methanol, 100/0 to 85/15, v/v) and precipitated from hexane (3.5 g, 75% yield) to give **6b**.

TLC R_f=0.79 (system B), 0.37 (system C); ^1H NMR ($CDCl_3$) δ 1.06 (s, 9H, $C(CH_3)_3$), 2.54 (m, 1H, H-2′, $J_{2',2''}$ 13.7 Hz, $J_{2',3'}$ 4.2 Hz), 2.85 (m, 1H, H-2″, $J_{2'',3'}$ 6.4 Hz), 3.41 (dd, 2H, H-5′and H-5″, $J_{5',5''}$ 5.1 Hz), 3.76 (s, 6H, OCH_3), 4.14 (q, 1H, $J_{4',5'}$ 1.5 Hz), 4.64 (bs, 1H, H-3′), 5.12 (s, 2H, CH_2OSi), 6.47 (t, 1H, H-1′, $J_{1',2'}$ 6.4 Hz), 7.19−7.82 (m, 14H, aromatic H), 8.11 (s, 1H, H-2), 8.67 (s, 1H, H-8), 9.39 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 19.1 ($C(CH_3)_3$), 26.7 ($C(CH_3)_3$), 40.0 (C-2′), 55.0 (OCH_3), 63.5 (C-5′), 63.8 (CH_2OSi), 71.9 (C-3′), 84.5 (C-4′), 86.2 (C-1′), 86.3 (Cq, DMT), 123.1 (C-C(O)), 112.9−135.5 (CH, aromatic) 132.4 and 132.9 (2×CSi, phenyl), 140.6 (CCH_2), 144.3 (Cq, phenyl), 149.2 (C-4), 151.2 (C-6), 158.3 (2×$COCH_3$), 165.9 (C=O).

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-2(*tert*-butyldiphenylsilyloxymethyl)-benzoyl deoxyadenosine 3′-*O*-2-cyanoethyl-*N*,*N*-(diisopropylamino)phosphoramidite (8)

To a solution of 5′-*O*-(4,4′-dimethoxytrityl)-6-*N*-2(*tert*-butyldiphenylsilyloxymethyl)-benzoyl deoxyadenosine (0.93, 1.0 mmol) and *N*,*N*-diisopropylethylamine (0.44 ml, 2.5 mmol) in dry dichloromethane (5.0 ml) was added (2-cyanoethyl)-*N*,*N*-diisopropylaminochlorophos-phite (0.3 g, 1.25 mmol). After stirring for 1 h at 20°C, the reaction mixture was diluted with dichloromethane (50 ml), washed with aqueous sodium hydrogen carbonate (20 ml, 0.5 M) and aqueous sodium chloride (20 ml, saturated). The organic layer was dried over magnesium sulfate, concentrated and coevaporated with toluene (10 ml) *in vacuo* to give an oil. The product was purified by column chromatography (15 g, hexane/ethyl acetate/triethylamine, 15/82.5/2.5, v/v/v) to afford **8** as a foam (1.05 g, 93% yield).

TLC R_f= 0.56 and 0.66 (system D); ^{31}P NMR (CH_2Cl_2) δ 149.1.

α-*N*-(2-nitrophenylsulfenyl)phenylalanyl tyrosine amide (11)

The dipeptide **11** was prepared from α-*N*-(2-nitrophenylsulfenyl)phenylalanine and tyrosine amide according to a literature procedure[7].

α-*N*-(2-nitrophenylsulfenyl)phenylalanyl tyrosine amide *O*-(2-cyanoethyl)-*N*,*N*-(diisopropylamino)phosphoramidite (13)

To a solution of dipeptide **11** (0.24 g, 0.5 mmol) in dry dichloromethane (3.0 ml), was added 2-cyanoethyl-*bis*-(*N*,*N*-diisopropylamino)phosphite (1.25 ml, 0.5 M in CH_2Cl_2) and subsequently 1-*H*-tetrazole (44 mg, 0.63 mmol). After stirring for 10 min, the mixture was diluted with dichloromethane (50 ml), washed with aqueous sodium hydrogen carbonate (20 ml, 0.5 M) and aqueous sodium chloride (20 ml, saturated). The organic layer was dried over magnesium sulfate and concentrated *in vacuo* in the presence of toluene (5 ml). The product was purified by column chromatography (10 g, hexane/ethyl acetate/triethylamine, 20/79/1, v/v/v) and concentrated *in vacuo* to give a yellow foam (0.25 g, 74% yield).

TLC R_f=0.55 (system E); ^{31}P NMR (CH_2Cl_2) δ 146.9.

Deprotection and purification of the nucleopeptide (15−18)

The column containing immobilized **15** was disconnected from the Synthesizer and treated with ammonia in methanol (2.0 ml, saturated) for 1 h at 20°C in a sealed tube. The column was centrifugated (5 min) and rinsed with methanol (2×2.0 ml). The combined methanol layers containing crude **16** were analized by reversed phase FPLC analysis (rt 24.9 min, see Fig. 1 under A). The methanol was evaporated and the residue was dissolved in pyridine (0.5 ml) and tetrabutylammonium fluoride (1.6 ml, pyridine/water, 1/1, v/v, 40 μmol) was added. After 2 h at 20°C, water (10 ml) was added, the mixture was extracted with dichloromethane (2×3 ml) and the aqueous phase was concentrated *in vacuo*. The product was redissolved in water, analized by reversed phase FPLC analysis (rt 14.2 min, see Fig. 1 under B) and concentrated *in vacuo*. The residue was dissolved in methanol (5 ml) and 2-mercaptopyridine (1 mg, 9 μmol) was added. After 24 h at 20°C, the methanol was removed by evaporation, the oil was taken up in water (20 ml) and washed with diethyl ether (2×5 ml) followed by dichloromethane (2×5 ml). The aqueous phase was concentrated *in vacuo*, dissolved in triethylammonium bicarbonate buffer (0.5 ml, 0.15 M) and purified by gelfiltration over a Sephacryl column suspended in

the same buffer. The purified product was passed over a SP-Sephadex C-25 column (sodium-form) to give **18** as the sodium salt (1.8 mg). The resulting aqueous solution was lyophilized and relyophilized from deuterium oxide (3×0.5 ml).
FPLC Rt **18** (reversed phase) 11.0 min; Rt **18** (anion exchange) 14.9 min; Rt **18** (gelfitration) 188.6 min.;
^{31}P NMR (D_2O, pD= 6.5, 2 μM) δ −3.88, −0.41; ^{1}H NMR (D_2O, pD 6.5, 2 μM) δ 1.66 (s, 3H, CH_3, dT), 1.88 (m, 2H, 2×H-2″, dT), 2.23 (m, 3H, 2×H-2′, H-3′, dT), 2.70 (dd, 1H, H-β), 2.76 (m, 4H, 2×H-2′ and 2×H-2″, dA, 2.95 (dd, 1H, H-β), 3.05 (d, 2H, 2×H-β), 4.03−4.40 (1H, 1×H-α, 4×H-5′, 4×H-5″ and 4×H-4′), 4.43 (t, 1H, 1×H-α, $J_{\alpha,\beta}$ 5.8 Hz), 4.49−4.92 (4H, 4xH-3′), 5.97 (dd, 1H, H-1′, $J_{1',2'}$ 5.9 Hz, $J_{1',2''}$ 8.8, dT), 6.12 (t, 1H, H-1′, $J_{1',2}$ 6.8 Hz, dT), 6.26 (dd, 2H, 2×H-1′ $J_{1',2'}$ 6.8 Hz, $J_{1',2''}$ 14.0 Hz, dA), 6.82 (m, 5H, H aromatic, Phe), 7.12−7.28 (m, 4H, H aromatic, Tyr), 7.32 (s, 1H, H-6, dT), 7.43 (s, 1H, H-6, dT), 8.00 (s, 1H, H-2, dA), 8.03 (s, 1H, H-2, dA), 8.12 (s, 1H, H-8, dA), 8.31 (s, 1H, H-8, dA).

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9. In order to prevent the unwanted β-elimination in de deblocking of **2** to give **1**, the commonly used N-acyl (*i.e.*, benzoyl or anisoyl protecting groups Y in **2**) were replaced in earlier studies (see ref. 7, 8) by the 2-nitrophenylsulfenyl (in case of d-adenosine or d-cytosine) or the di-n-butylaminomethylene group (in the case of d-guanosine).
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