Stepwise Solid-Phase Synthesis of the Nucleopeptide Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH

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Received November 2, 1993•

The nucleopeptide Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH, with a phosphodiester bond between the side chain hydroxyl group of a serine residue and the 3′ end of a trinucleotide, has been synthesized by a stepwise procedure. The peptide was first assembled on an insoluble matrix and the oligonucleotide chain elongation was then carried out at the serine hydroxyl group of the resin-linked peptide by the phosphite triester approach using standard phosphoramidite derivatives. Mild basic conditions were used for the final deprotection of the permanent protecting groups.

Peptide–oligonucleotide conjugates with a covalent phosphodiester bond between the terminal hydroxyl group of a DNA or RNA fragment and a side chain hydroxyl group of a peptide are referred to as nucleopeptides. The synthesis of nucleopeptides with a phosphate group linking two hydroxyl groups has been carried out by all the methodologies available to create phosphodiester bonds. Thus, covalent linkages between the 5′-hydroxyl of a nucleoside and the side chain hydroxyl of an amino acid have been formed in solution by either the phosphotriester,[1] the H-phosphonate,[2] or the phosphite triester[1c] methods and on an insoluble matrix using the phosphite triester approach.[3]

All the syntheses described so far have been carried out by a convergent approach in which the two different components of the molecule, peptide and oligonucleotide, are first assembled separately and then linked through the phosphate bond. Although the combination of the phosphite triester approach and the solid-phase methodology has allowed nucleopeptides of a certain complexity to be obtained, the overall procedure is still lengthy and troublesome. On the one hand, the protecting groups used often have to be removed under different conditions, carefully chosen so as not to degrade the target molecule; on the other hand, the purification, phosphitylation, and coupling of the protected peptide onto the oligonucleotide-resin may be complicated by the insolubility of protected peptides in most organic solvents.

In the search for a simple and general method for the synthesis of nucleopeptides, we have explored the viability of a stepwise solid-phase approach. Conjugates[4] with different types of union between the peptide and the oligonucleotide have been assembled on a solid support, but this methodology has not, as yet been used to obtain nucleopeptides. We now report on the stepwise synthesis of nucleopeptide Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH[5], in which the 3′ end of an oligodeoxynucleotide and the side chain of a residue of serine are linked through a single phosphodiester bond. The term "nucleopeptide" is used here to describe mixed oligomers in which the peptide is linked to either end of the oligonucleotide through a phosphodiester bond and could thus be used to refer to either peptide-phosphate-5′-oligonucleotide structures as in the nucleoproteins associated with the replication of the genetic material of some viruses[6] or to peptide-phosphate-3′-oligonucleotide structures such as those formed when the linking number of DNA is altered by the action of topoisomerases.[7]

In our stepwise strategy, the peptide is first assembled on the appropriately functionalized solid matrix, and the oligodeoxynucleotide chain elongation is then carried out at the side chain hydroxyl group of the serine residue by the phosphite triester approach. Thus, use of standard 3′-phosphoramidite nucleoside derivatives affords a 3′ nucleopeptide regioisomer, while nucleopeptides with the natural linkage would be accessible from 5′-phosphoramidite nucleoside derivatives. The success of this synthetic strategy obviously depends upon both (i) high yields in the coupling of the nucleoside phosphoramidite

• Abstract published in *Advance ACS Abstracts,* April 1, 1994.

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(5) Abbreviations: aa, amino acid; bz, benzoyl; CNE, cyanoethyl; DMT, 4,4′-dimethoxytrityl; EDIA, *N*-ethyl-*N*,*N*-diisopropylamine; HOBt, 1-hydroxybenzotriazole; ibu, isobutyryl; N, deoxynucleoside; oNPT, 5-(o-nitrophenyl)tetrazole; P, polystyrene-*co*-1%-divinylbenzene; Phac, phenylacetyl; TBAF, tetrabutylammonium fluoride; TCA, trichloroacetic acid; Tcp, 2,4,5-trichlorophenyl.

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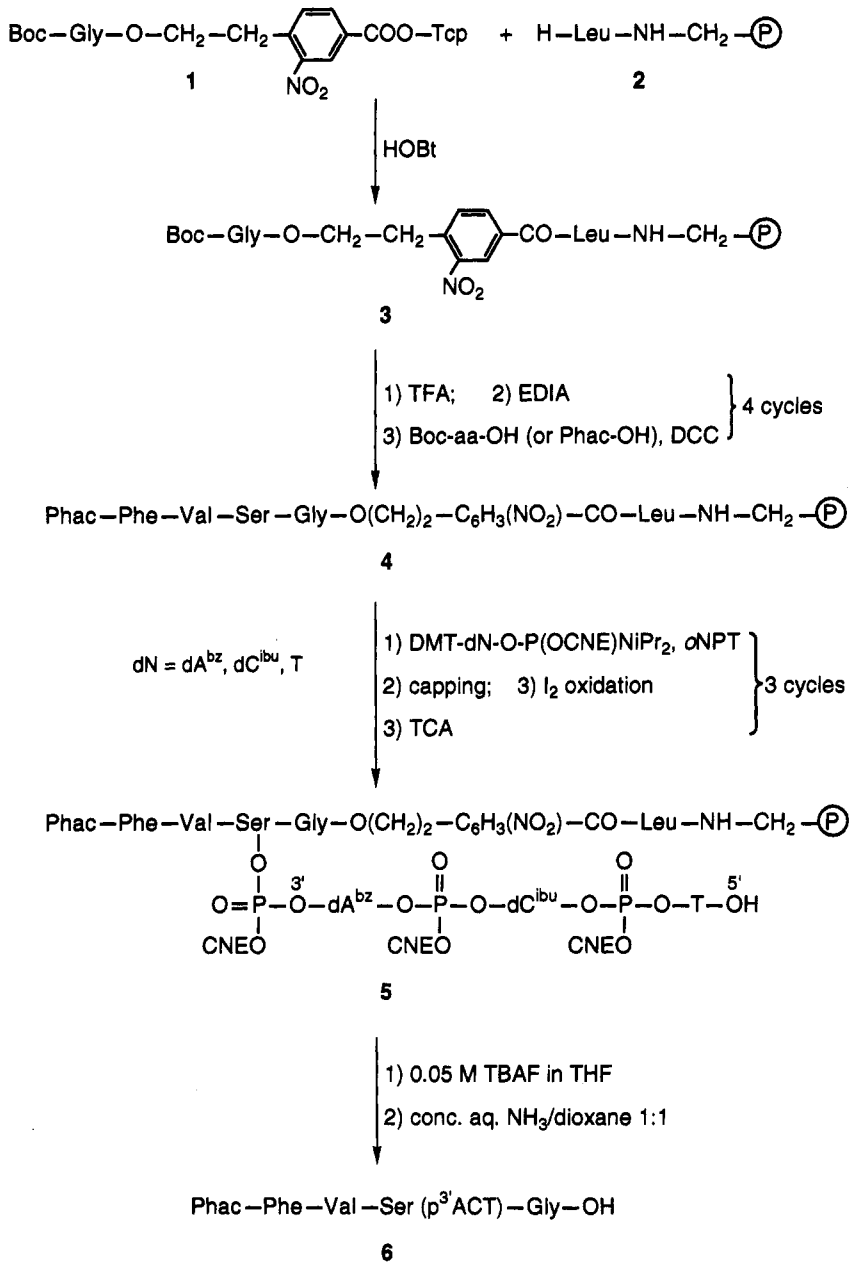

Figure 1. Synthesis of nucleopeptide Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH.

derivatives onto the resin-linked peptide, and (ii) the correct choice of protecting groups and the conditions under which they are removed. The synthesis scheme is outlined in Figure 1.

The standard acid-labile Boc and DMT groups were used for the temporary protection of the α-amino- and the 5′-hydroxyl groups during the peptide and oligonucleotide elongation cycles, respectively. All the permanent protecting groups were chosen so as to be eliminated under basic conditions mild enough not to promote the cleavage of the peptide–oligonucleotide phosphate bond by β-elimination:[8,9] a 2-(nitrophenyl)ethyl ester bond[10] to link the C-terminal amino acid to the resin, 2-cyanoethyl groups to protect the phosphate functions, and benzoyl and isobutyryl groups to carry out the protection of the exocyclic amines of deoxyadenosine and deoxycytosine, respectively.

The C-terminal amino acid was anchored to the solid matrix by a two-step process: the active ester derivative 1 was first obtained by reaction of Boc-glycine with 2,4,5-trichlorophenyl 4′-(hydroxyethyl)-3′-nitrobenzoate[10a] in the presence of DCC and DMAP, and this was then coupled onto leucyl(aminomethyl)polystyrene 2 (leucine was used as internal reference amino acid) in the presence of HOBt. Unreacted resin-bound amines were acetylated and the stepwise elongation of the peptide was carried out on Boc-glycine-resin 3 using standard procedures. Boc-serine was incorporated without protection of the side chain hydroxyl group, and after incorporation of phenylalanine, the N-terminus was blocked by reaction with phenylacetic acid and DCC (the phenylacetyl group was chosen in order to facilitate the analysis of the peptide both by HPLC and ^{1}H-NMR). The amino acid composition of the peptide-resin after acid hydrolysis was in accord with the expected

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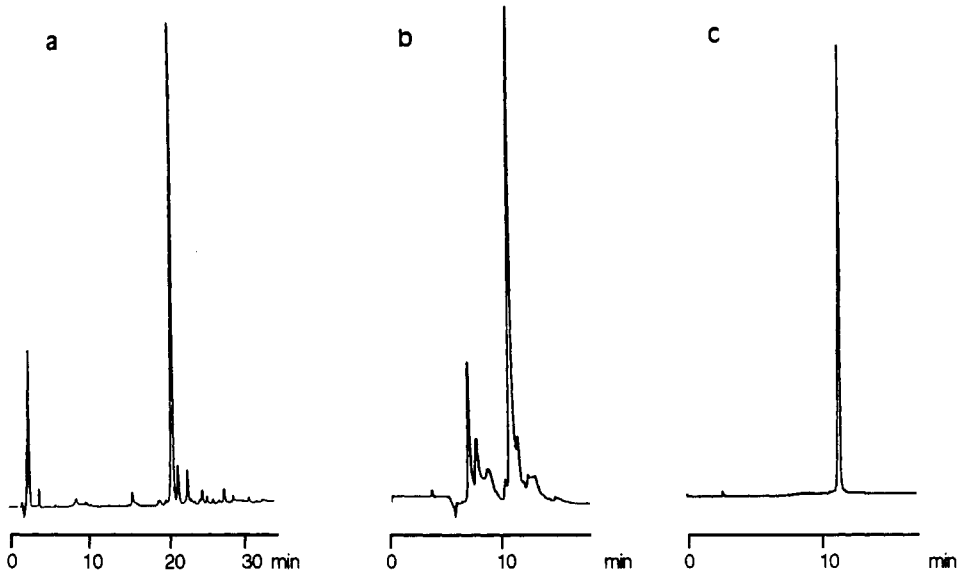

Figure 2. HPLC profiles of (a) crude peptide Phac-Phe-Val-Ser-Gly-OH, (b) crude nucleopeptide **6** and (c) purified nucleopeptide Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH. See Experimental Section for analysis conditions and retention times.

values, and the peptide obtained after cleavage of an aliquot of peptide-resin with TBAF in THF and purification through Sephadex LH-20 had the correct mass. The HPLC trace of the crude peptide is shown in Figure 2.

The stepwise elongation of the trinucleotide at the serine side chain hydroxyl group of the resin-linked peptide **4** was carried out by the phosphite triester approach (18-μmol scale). The cycle program was adapted to synthesis on a polystyrene support[11] and commercially available 5′-DMT-dN-3′-phosphoramidites (dN: dA^{bz}, dC^{ibu}, T) were activated and coupled in the presence of 5-(*o*-nitrophenyl)-tetrazole.

Treatment of nucleopeptide-resin **5** with 0.05 M TBAF in THF (30 min, room temperature) quantitatively detached the nucleopeptide from the solid matrix (as determined by amino acid analysis of the resin after this treatment). Final deprotection was achieved by means of an 18-h treatment with concd aqueous NH_3/dioxane 1:1 at room temperature, and the target nucleopeptide **6** was obtained after purification by ion-exchange chromatography on DEAE-Sephadex in 40% overall yield after synthesis, cleavage, deprotection, and purification. Purified **6** was shown to be homogeneous by reversed-phase HPLC (see Figure 2) and different from the oligonucleotide-3′-phosphate (TpCpAp) which would be formed were the molecule to be degraded by β-elimination.[12] Phac-Phe-Val-Ser(p$^{3'}$ACT)-Gly-OH (**6**) was characterized by ^{31}P-NMR and ^{1}H-NMR, mass spectrometry, amino acid analysis after acid hydrolysis, and nucleoside composition after enzymatic digestion (see data in the Experimental Section).

In summary, solid-phase stepwise synthesis has been shown to be efficacious for the straightforward synthesis of nucleopeptides, and a hybrid molecule with a phosphodiester bond linking the hydroxyl groups of a nucleoside and a residue of serine has been obtained by this method for the first time. The peptide was assembled on a polystyrene matrix without protection of the side chain of the serine residue, and the analysis of the peptide showed that very pure material was anchored to the resin. The oligonucleotide chain elongation at the serine hydroxyl group of the resin-linked peptide was successfully carried out by the phosphite triester approach using commercially available phosphoramidites. The combination of protecting groups used proved to be convenient and the polystyrene solid support, suitable for the synthesis of both peptides and oligonucleotides, was compatible with all the reagents used throughout.

The overall strategy is considerably simpler than a convergent approach, and the final deprotection and cleavage conditions have been fine-tuned in order to allow the synthesis of nucleopeptides with a base-labile serine–nucleoside phosphate diester bond in good yield. Work is in progress to extend this synthetic strategy to the preparation of more complex nucleopeptides with different amino acid–nucleoside phosphate linkages, which may be used in structural studies, as models to study the interaction of antitumor drugs with DNA–topoisomerase complexes,[7b] or in antiviral therapy.[14] If the peptide linked to the 3′ end were able to inhibit or reduce the action of 3′ exonucleases (which seem to be the most deleterious agents of degradation[15]), 3′-nucleopeptides would be particularly interesting as antisense inhibitors of gene expression.

Experimental Section

General Information. Boc-L-amino acids and aminomethyl polystyrene-*co*-1%-divinylbenzene were obtained from Bachem Feinchemikalien AG and Novabiochem AG. DMT-dC^{ibu}-, DMT-dA^{bz}-, and DMT-T-3′-phosphoramidites were purchased from Applied Biosystems. Peptide synthesis was performed manually in a polypropylene syringe fitted with a polyethylene disc and the stepwise elongation of the trinucleotide at the serine side chain hydroxyl group of the resin-linked peptide was performed on a 380B Applied Biosystems synthesizer following standard

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(12) Trinucleotide-3′-phosphate TpCpAp was obtained as described in ref 10a; t_R 7.8 min (the same HPLC analysis conditions as for the target nucleopeptide **6**).

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procedures with modifications rendering the phosphite triester methodology compatible with the use of polystyrene.[11] Dry acetonitrile (ACN) was obtained by distillation and storage over CaH_2. Dichloromethane (DCM) was neutralized and dried by passing through basic alumina and storage over CaH_2. Dry THF was obtained directly by distillation over sodium metal in the presence of benzophenone. DMF was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4-Å molecular sieves. All other solvents not required dry were distilled prior to use. DEAE-Sephadex and LH-20 Sephadex were from Pharmacia. 2 M Triethylammonium bicarbonate (TEAB) buffer was prepared by passing a stream of carbon dioxide through a solution of Et_3N in water until a neutral pH/solution was obtained. Reversed-phase HPLC analyses were performed on Spherisorb-ODS columns (10 μm, 250 × 4.6 mm, 1 mL/min, detection wavelength: 260 nm) using linear gradients of 0.045% TFA in water and 0.036% TFA in ACN for peptide analyses (system RPA) and 0.01 M aqueous triethylammonium acetate and ACN (system RPB) or ACN/H_2O (1:1) (system RPC) for nucleoside, oligonucleotide, and nucleopeptide analyses. Amino acid analyses of the peptidyl resins, peptides, and nucleopeptides were performed after hydrolysis in 12 M HCl/propionic acid (1:1) at 150 °C for 60–90 min.

Boc-Gly-O(CH_2)$_2$-$C_6H_3(NO_2)$-CO_2-Tcp (1). DCC (618 mg, 3 mmol) and a catalytic amount of DMAP (12 mg, 0.1 mmol) were added to a solution of 2,4,5-trichlorophenyl 4′-(2-hydroxyethyl)-3′-nitrobenzoate[10a] (390 mg, 1 mmol) and Boc-Gly-OH (526 mg, 3 mmol) in dichloromethane (5 mL), and the resulting mixture was stirred for 30 min at room temperature. The *N,N′*-dicyclohexylurea formed was removed by filtration and the filtrate was diluted with dichloromethane (25 mL), washed with 5% aqueous $NaHCO_3$ (2 × 25 mL) and saturated brine (25 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography on silica gel eluting with hexanes/ethyl acetate 3:1 to yield a yellow foam (374 mg, 70% yield): R_f ($CHCl_3$/MeOH/AcOH 73:25:2) 0.80; ^{1}H-NMR ($CDCl_3$, 200 MHz) δ 8.75 (1H, d, *J* 1.8 Hz), 8.44 (1H, dd, *J* 1.8 Hz, 6.0 Hz), 7.61 (2H, m), 7.46 (1H, s), 5.16 (1H, m), 4.51 (2H, t, *J* 6.0 Hz), 3.88 (2H, d, *J* 6.0 Hz), 3.38 (2H, t, *J* 6.0 Hz), 1.45 (9H, s); ^{13}C-NMR ($CDCl_3$, 50 MHz) δ 170.8, 162.0, 156.2, 150.3, 146.0, 139.4, 134.6, 134.0, 131.7, 127.3, 125.7, 80.6, 64.5, 42.8, 33.0, 28.8; FAB-MS (nitrobenzylic alcohol matrix, Xe, positive mode) *m/z* 549 $[M + H^+]^+$, 449 $[M - Boc + 2H^+]^+$, 374 $[M - (Boc\text{-}Gly\text{-}O)]^+$.

H-Leu-NH-CH_2-P (2). Boc-Leu-OH was anchored to (aminomethyl)polystyrene (previously washed with DCM, 30% TFA/DCM, DCM, 5% EDIA/DCM, and DCM) following the same standard procedure described below for the solid-phase peptide assembly. The α-amino group was deprotected by a 30% TFA treatment, neutralized with 5% EDIA in DCM, and washed. The resulting aminoacyl-resin had a substitution degree of 0.30 mmol/g.

Boc-Gly-O(CH_2)$_2$-$C_6H_3(NO_2)$-CO-Leu-NH-CH_2-P (3). Boc-Gly-O(CH_2)$_2$-$C_6H_3(NO_2)$-CO_2-Tcp (1) (206 mg, 0.38 mmol), HOBt·H_2O (61 mg, 0.45 mmol), and H-Leu-NH-CH_2-polystyrene (2) (1 g) were suspended in the minimum amount of DMF in a syringe fitted with a disc, and the mixture was gently stirred for 15 h at room temperature. The resin was then washed and dried, and the unreacted amino groups were blocked by treatment with a mixture of acetic anhydride (0.47 mL, 5 mmol) and EDIA (0.87 mL, 5 mmol) in DMF for 30 min. Once the resin was washed and dried, the loading of glycine was determined by amino acid analysis and was found to be 0.18 mmol/g (Gly/Leu = 0.62).

Phac-Phe-Val-Ser-Gly-O(CH_2)$_2$-$C_6H_3(NO_2)$-CO-Leu-NH-CH_2-P (4). The peptide was assembled manually on Boc-Gly-resin 3 using a standard protocol that comprises an acidolytic removal of the Boc protecting group with 30% TFA/DCM (5 + 25 min), neutralization of the α-amino group with 5% EDIA/DCM, and a 1-h DCC-mediated coupling of the corresponding amino acid (3 equiv of both Boc-amino acid and DCC in DCM or mixtures of DCM and DMF depending on the solubility of the amino acids). A qualitative ninhydrin test was used to monitor the synthesis. Boc-serine was introduced with the side chain unprotected. After incorporation of phenylalanine, the N-terminus was blocked by reaction with phenylacetic acid and DCC in the same conditions as the amino acid derivatives. The amino acid composition was determined by amino acid analysis after acid hydrolysis and was found to be in accord with the expected values: Phe 0.96, Val 1.00, Ser 0.72, Gly 1.04; peptide/Leu = 0.61.

An aliquot of peptide-resin was treated with a 0.05 M solution of TBAF in dry THF (100 equiv with respect to the peptide-resin) for 30 min. After addition of 10% AcOH, the filtrate was analyzed by reversed-phase HPLC (system RPA, linear gradient from 10 to 80% B in 30 min): t_R of peptide Phac-Phe-Val-Ser-Gly-OH: 20.0 min, see Figure 2. The peptide was purified by gel filtration through Sephadex LH-20 (75 × 2 cm column) eluting with MeOH (0.5 mL/min, detection at 254 nm) and characterized by FAB-MS (nitrobenzyl alcohol matrix, Xe, positive mode) *m/z* 549.8 $[M + Na^+]^+$, 527.6 $[M + H^+]^+$ and ^{1}H-NMR (CD_3OD, 200 MHz) δ 7.20–7.10 (10H, m), 4.55 (1H, m), 4.25 (1H, m), 4.00–3.80 (3H, m), 3.69 (2H, s), 3.22, 2.78 (2H, m), 2.12 (1H, m), 0.92 (6H, m).

Protected Nucleopeptide-resin (5). The stepwise elongation of the trinucleotide at the serine side chain hydroxyl group of peptide-resin 4 (100 mg, 18-μmol scale) was carried out on a 380 B Applied Biosystems DNA synthesizer following the standard protocol with small modifications.[11] The main steps of the synthesis cycle are the following: (i) DMT elimination with 3% TCA/DCM (3 × 3 min); (ii) neutralization with 2% EDIA/DCM (2 min); (iii) coupling of 5′-DMT-nucleoside-3′-phosphoramidites (800 μL of a 0.25 M solution in DCM) in the presence of (*o*-nitrophenyl)tetrazole (800 μL of a 1 M solution in THF) for 40 min; (iv) capping of unreacted hydroxyl groups with acetic anhydride and *N*-methylimidazole; (v) oxidation of the phosphite groups to phosphates with aqueous iodine.

(*o*-Nitrophenyl)tetrazole was obtained as described[13] in a 70% yield; mp 162–164 °C; IR (KBr) 3100–2500, 1520, 1365 cm^{-1}; ^{1}H-NMR (CD_3COCD_3, 200 MHz) δ 8.18 (1H, m), 7.98 (4H, m).

Phac-Phe-Val-Ser(p^3'ACT)-Gly-OH (6). Nucleopeptide-resin 5 (20 mg, approximately 2 μmol) was placed in a polypropylene syringe fitted with a polyethylene disc and treated with 0.05 M TBAF in dry THF (4 mL, 100 equiv) for 30 min after which time the resin was filtered and washed with MeOH (3 × 5 mL). The filtrates were pooled and evaporated after addition of 10% aqueous AcOH. The cleavage yield was determined by amino acid analysis of the resin after acid hydrolysis and was found to be quantitative. Final deprotection of the product was achieved by means of an 18-h treatment with concd aqueous NH_3/dioxane 1:1 (10 mL) at room temperature in a capped vessel. The solution was evaporated, and the resulting crude was dissolved in water and filtered to remove insoluble products. Reversed-phase HPLC analysis (system RPB, linear gradient from 0 to 50% of B in 20 min) of the crude nucleopeptide showed a major product (t_R 10.8 min, see Figure 2). Purification was accomplished by anion-exchange chromatography on DEAE-Sephadex (30 × 1 cm column) eluting with a gradient of 0.4 M to 1 M TEAB buffer (0.5 mL/min, detection at 254 nm). The fractions containing the desired product were pooled and lyophilized and shown to be homogeneous by reversed-phase HPLC (Figure 2). Nucleopeptide 6 was obtained in a 40% overall synthesis, cleavage, deprotection, and purification yield (24 OD_{260}) and was characterized as follows:

Amino acid analysis after acid hydrolysis of an aliquot of purified 6 (0.5 OD_{260}): Phe 1.00, Val 0.91, Ser 0.44, Gly 1.09. The serine value increased to 0.92 on acid hydrolysis in a vacuum degassed sealed tube for 24 h at 110 °C.

Nucleoside composition after enzymatic digestion (0.5 OD_{260}) with calf spleen phosphodiesterase (EC 3.1.16.1, Sigma; 0.2 M NH_4AcO, pH 5.4, 6 h, 36 °C) and reversed-phase HPLC analysis (system RPC, linear gradient from 0 to 100% B in 20 min): deoxycytidine (t_R 8.4 min), deoxycytidine-3′-phosphate (t_R 7.7 min), thymidine (t_R 10.1 min), thymidine-3′-phosphate (t_R 8.6 min), and a fifth product of undetermined structure (t_R 14.4 min; presumably a deoxyadenosine derivative in which the nucleoside is linked to the tetrapeptide). Upon treatment of the mixture with 1 M aqueous piperidine for 1 h at 50 °C, this latter product was transformed into deoxyadenosine-3′-phosphate (t_R 9.5 min). The different products (among which the presence of nucleosides might be explained by phosphatase contamination of the enzyme used) were identified by comparison with authentic samples of nucleosides and nucleoside-3′-phosphates. In an alternative

experiment, an aliquot of nucleopeptide 6 was first treated with 1 M NaOH for 2 at 50 °C, and after neutralization with glacial AcOH and lyophilization, was digested with a mixture of snake venom phosphodiesterase and alkaline phosphatase. HPLC analysis (system RPC, 10 min isocratic 5% B, linear gradient from 5 to 60% B in 10 min, and linear gradient from 60 to 100% B in 5 min) showed a mixture of deoxycytidine (t_R 4.9 min), thymidine (t_R 12.6 min), and deoxyadenosine (t_R 17.5 min) in relative proportions of 0.91, 1.01, and 1.08, respectively.

^{1}H-NMR (Na^+ salt, obtained after chromatography through Dowex 50Wx4 and lyophilization from D_2O; D_2O, 500 MHz) δ 8.21 (1H, s); 7.91 (1H, s); 7.38–7.36 (2H, m); 7.11, 6.94, 6.87 and 6.73 (10H, m); 6.12, 6.00, 5.92 (3H, m); 5.76 (1H, d, J 8 Hz); 5.00–3.50 (10 H); 2.80–2.00 (7H, m); 1.67 (3H, s); 0.72, 0.70 (6H, 2d) ppm.

^{31}P-NMR (Na^+ salt, D_2O, 121 MHz, external reference $P(OCH_3)_3$) δ 0.85, 0.65, 0.46 ppm.

FAB-MS (glycerol/TFA/H_2O matrix, Xe, negative mode) m/z 1432 $[M - H]^-$ (expected mass for M, $C_{56}H_{71}O_{25}N_{14}P_3$ 1433.2) and electrospray-MS (eluent ACN/H_2O 1:1, injection: 25 μL of a solution containing 1 OD_{260}/mL, negative mode) m/z 477 $[M - 3H]^{3-}$, 716 $[M - 2H]^{2-}$, 727 $[M - 3H + Na]^{2-}$, 738 $[M - 4H + 2Na]^{2-}$.

Acknowledgment. The authors are grateful for the use of the facilities of the "Servei de RMN de la Universitat de Barcelona". This work was supported by funds from the DGICYT (grant PB91-0270).