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A CONVENIENT APPROACH TOWARD THE PREPARATION OF NUCLEOPEPTIDES

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Abstract- The use of the 2-nitrophenylsulfenyl group for the protection of the N-terminus of peptides and the exocyclic-amino function of deoxyadenosine will be illustrated in the assemblage of the nucleopeptides H-Phe-Tyr(pATAT)-NH_2 and H-Ala-Ser(pATAT)-Ala-OAllyl *via* phospho- and phosphitetriester intermediates.

Nucleoproteins (peptides) are naturally occurring polymers in which the hydroxy groups of the L-amino acids serine, threonine or tyrosine in the protein (peptide) part are covalently attached *via* a phosphodiester linkage to the 5'-end of nucleic acids (DNA or RNA[1]). The introduction of the phosphodiester bond between the nucleic acid and peptide units can be most conveniently achieved by using one of the several phosphorylation methods thus far developed in nucleic acids chemistry. Indeed, in earlier studies on the preparation of nucleopeptides we showed that a phosphotriester[2] or a H-phosphonatediester[3] approach could be successfully applied for the formation of the required phosphodiester bond. However, this synthetic route gave only access to nucleopeptides, the nucleic acids moiety of which consisted solely of nucleobases lacking exocyclic-amino functions (*e.g.*, thymidine or uridine). The reason for this limitation is as follows. Basic hydrolysis of the commonly used N-acyl groups for the protection of the exocyclic-NH_2 functions of the purine and pyrimidine bases, adenine, guanine and cytosine is not compatible with the presence in a nucleopeptide of serininyl (threoninyl)nucleotidyl phosphotri- or diester linkages: in both cases β-elimination results[4] in the formation of dehydro-peptide derivatives and the corresponding nucleic acids 5'-phosphates. Further, basic deblocking conditions may have a deleterious effect on the chiral integrity of the peptide part in the nucleopeptide.

We now report that protection of the exocyclic-amino function of adenosine with the 2-nitrophenylsulfenyl group enabled us to prepare the nucleopeptide fragments, H-Phe-Tyr(pATAT)-NH_2 and H-Ala-Ser(pATAT)-Ala-OAllyl.

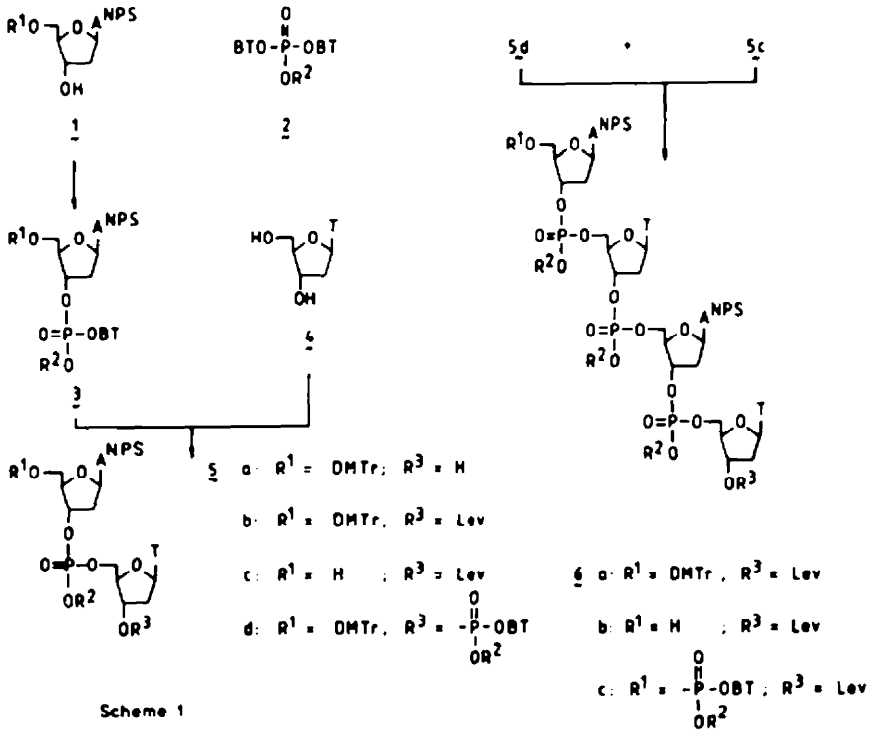
In a recent communication it was reported[5] that the 2-nitrophenylsulfenyl (NPS) group was suitable for the protection of the exocyclic-amino group of deo..., and ribonucleosides. Thus, apart from its ease of introduction, it was illustrated that the NPS-group survived commonly used phosphorylation methods, and that its removal could be effected by simple thiolysis. Another interesting feature was that the presence of an NPS-group increased the stability of the N-glycosidic bond in purine deoxynucleosides towards acidic hydrolysis (depurination). The above mentioned favorable properties of the NPS-group, together with the interesting observation that this N-protecting group was compatible with the use of the acid-labile 4,4'-dimethoxytrityl (DMT) group for the protection of the 5'-hydroxyl function in DNA, urged us to employ the NPS protecting-group-strategy in the synthesis of the

nucleopeptide fragment H-Phe-Tyr(pATAT)-NH_2 (8d). The steps involved in the assemblage of this nucleopeptide, which is a naturally occurring fragment[6] of the nucleoprotein formed in the initial stage of the rolling circle replication of double stranded circular DNA of bacteriophage ϕX 174 by gene A protein, is illustrated in Scheme 1 and 2.

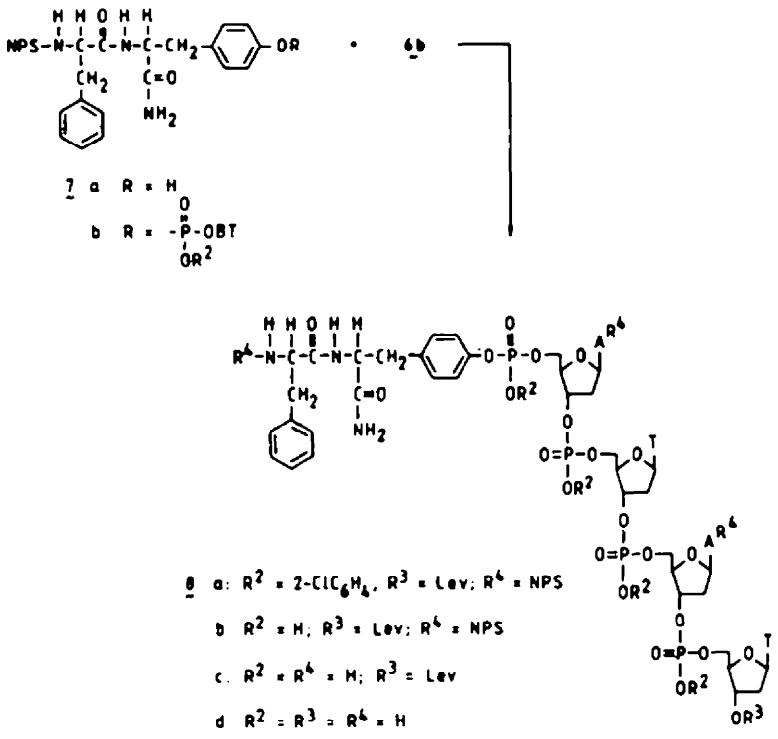
The preparation of tetramer 6b having a free 5'-hydroxyl group is outlined in Scheme 1. Phosphorylation of 5'-O-dimethoxytrityl-6-N-(2-nitrophenylsulfenyl)adenosine (1), obtained by a slight modification of a literature procedure[5], with the bifunctional reagent O-(2-chlorophenyl)-O,O-*bis*(1-benzotriazolyl)phosphate[7] (2) gave intermediate 3. Regioselective coupling of 3 with a slight excess of thymidine 4 afforded key dimer 5a in a yield of 70%. The latter was quantitatively converted into the 3'-O-levulinoyl derivative 5b by acylation with levulinoic acid anhydride[8]. Acidolysis[9] of the 4,4'-dimethoxytrityl group (R^1) from 5b gave dimer 5c having a free 5'-hydroxyl group. On the other hand, phosphorylation of 5a with 2 yielded 5d still having at the 3'-end one activable benzotriazolyl (BT) phosphate function. N-Methylimidazole-mediated condensation of dimer 5d with dimer 5c, resulted in the fully-protected tetramer 6a, which was detritylated, to give 6b in an overall yield of 87% (based on 5a).

The coupling of dipeptide NPS-Phe-Tyr-NH_2 (7a) with tetramer 6b is depicted in Scheme 2. Dipeptide 7a, prepared by HOBT/DCCI-mediated condensation[10] of NPS-Phe-OH[11] with H-Tyr-NH_2[12] was phosphorylated with 2 to yield intermediate 7b. The latter was then coupled, as mentioned before, with tetramer 6b to furnish fully-protected nucleopeptide 8a in an excellent yield. The favourable outcome of the oxybenzotriazolide phosphotriester approach indicates that the concomitant release of 1-hydroxybenzotriazole in the two-step phosphorylation procedure is compatible with the presence of the NPS-groups[13].

Complete deblocking of nucleopeptide 8a was effected in three consecutive steps. The 2-chlorophenyl (R^2) groups were removed with oximate-ions[14] to give, after work-up and purification by Sephadex LH-20 chromatography, partially-protected 8b. The NPS (R^4) groups were then removed under very mild conditions with tri-n-butylphosphine in dioxane-water. The same procedure has been used before for the removal of the 3-nitro-2-pyridinesulfenyl group from cysteine derivatives[15]. In this respect it is interesting to note that the reaction of tri-n-butylphosphine with 8b results in the formation of a yellow non-charged NPS derivative



Scheme 1



Scheme 2

(presumably the corresponding disulfide) and the simultaneous discoloration of the starting product, the progress of which can easily be judged by TLC-analysis. Finally, hydrazinolysis of 8c yielded, after work-up and purification, homogeneous 8d. The ^{1}H-, ^{13}C- and ^{31}P NMR spectra of 8d (Na^{+}-salt) thus obtained were in full accord with its proposed structure (see Fig. 1). The above mentioned three-step deblocking procedure was also executed by using in the first step tetra-n-butylammonium fluoride instead of the oximate procedure. The net result of this alternative step was a lower yield of 8b which is mainly due to the non-selectivity of the fluoride-ions promoted cleavage of the 2-chlorophenyl groups[16].

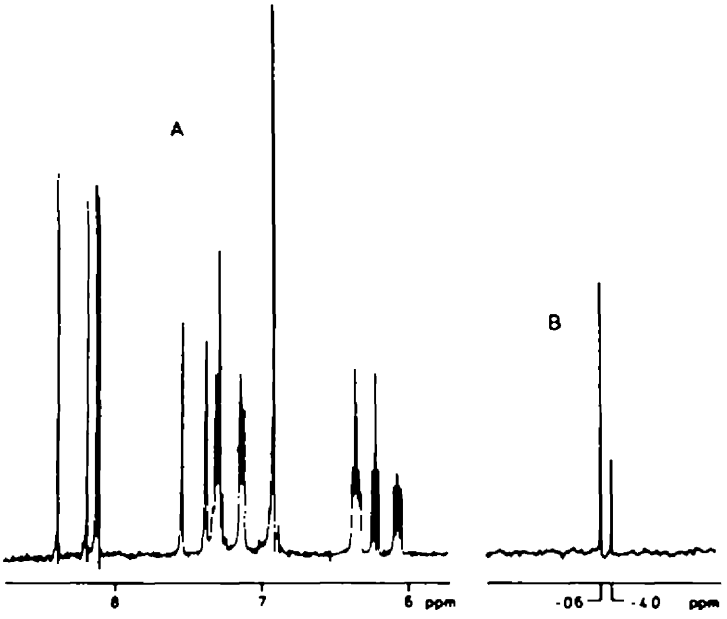
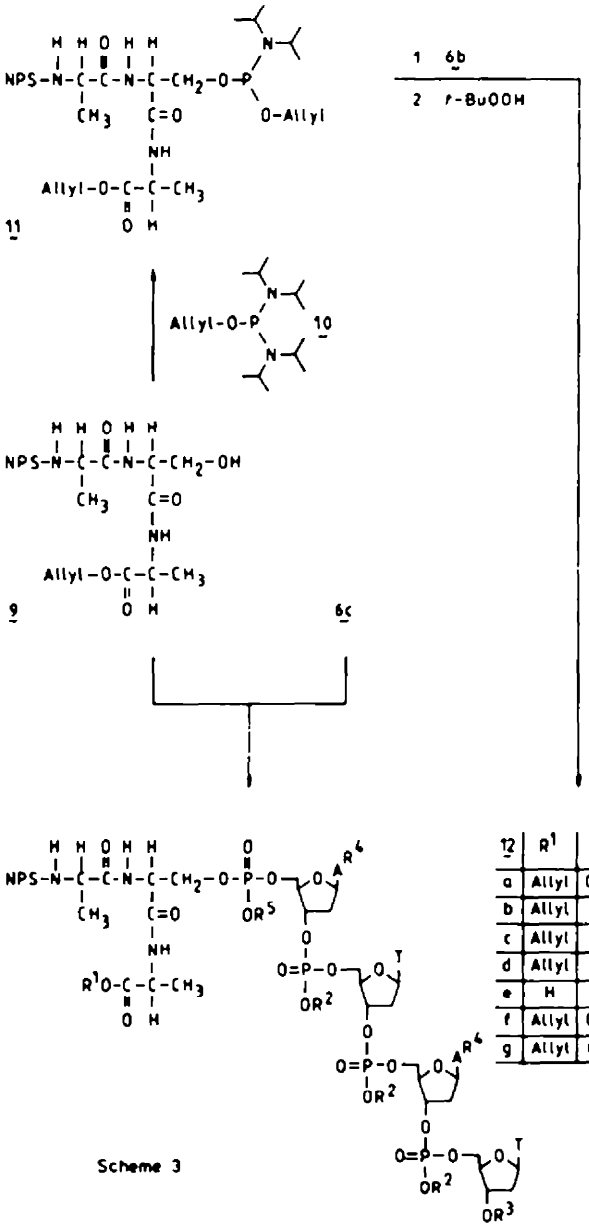


Figure 1. A. Low-field ^{1}H NMR spectrum of fully deprotected nucleopeptide H-Phe-Tyr(pATAT)-NH_2 (8d) showing resonances of: H-1' protons of the deoxyriboses (6 ppm region); H-6 of the thymines and aromatic protons of Tyr and Phe (7 ppm region); H-2 and H-8 protons of the adenines (8 ppm region). B. ^{31}P NMR spectrum of 8d showing two resonances in the ratio 1:3. The resonance at -4.0 ppm may be attributed to the phosphodiester bond between the phenolic hydroxyl group of Tyr and the 5'-end of the tetramer.

The successful assemblage and deblocking of nucleopeptide 8d stimulated us to prepare the nucleopeptide H-Ala-Ser(pATAT)-Ala-OH (12c in Scheme 3). To achieve our goal two different phosphorylation methods for the coupling of protected tripeptide 9 with tetramer 6b were explored. Further, the highly advocated[17] allyl-group was applied for the protection of the C-terminus of alanine in the tripeptide.

In a first approach tripeptide 9, obtained by condensing NPS-Ala-Ser-OH with H-Ala-OAllyl[17a], in the presence of isobutyl chloroformate and N-methylmorpholine, was coupled with intermediate 6c prepared *in situ* by phosphorylation of 6b with 2 (see Scheme 1). Work-up and purification afforded homogeneous 12a, as judged by TLC-analysis and ^{31}P NMR spectroscopy, in a yield of 64% (based on 6b). The alternative assemblage route (see preparation of 8a in Scheme 2) consisting of phosphorylation of tripeptide 9 with 2 followed by the addition of tetramer 6b proved to be not successful. In this particular case, the primary hydroxyl group of the serine moiety in 9 reacted two times with 2 to give a considerable amount of the undesired symmetrical product. On the other hand, the formation of this unwanted product



12	R^1	R^2	R^3	R^4	R^5
a	Allyl	ClC_6H_4	Lev	NPS	$Cl_2C_6H_3$
b	Allyl	H	Lev	NPS	H
c	Allyl	H	Lev	H	H
d	Allyl	H	H	H	H
e	H	H	H	H	H
f	Allyl	ClC_6H_4	Lev	NPS	Allyl
g	Allyl	ClC_6H_4	Lev	NPS	H

Scheme 3

could be eliminated by the following phosphite-triester method. To a solution of tripeptide 9 in acetonitrile was added equimolar amounts of 1-H-tetrazole and the phosphitylating reagent 10 which was prepared by a slight modification of a literature procedure[18]. TLC-analysis, after 30 min at 20°C, indicated the absence of 9 and the presence of a higher-running product (presumably 11). An equimolar amount of tetramer 6b together with 1-H-tetrazole were now added to the reaction mixture followed, after 18 h at 20°C, by oxidation of the intermediate formed phosphitetriester with t-butyl hydroperoxide[19]. Work-up, after 30 min at 0°C, and purification furnished nucleopeptide 12f in a yield of 90%. Deblocking of 12a to give 12d still having the allyl group at the C-terminus of alanine was performed by the second protocol mentioned earlier for the removal of the protecting groups from 8a. Unfortunately, any attempt to remove the remaining allyl group (R^1) in 12d to yield 12c using well-established procedures involving noble metal catalysts[17] failed.

The above described phosphitetriester approach has, apart from the high coupling efficiency, the additional advantage that the allyl-phosphate protecting group (R^5) of the extremely base-labile nucleotidyl-peptidyl phosphotriester bond in 12f can be quantitatively removed in pyridine-water. Thus monitoring of this process by ^{31}P NMR showed, after 48 h at 20°C, complete disappearance of one phosphorus resonance and the reappearance of a characteristic phosphodiester resonance. Further deblocking of 12g, in the same fashion as mentioned for 12a, afforded homogeneous 12d. The integrity of 12d, obtained by the two different routes, was unambiguously ascertained by ^1H-, ^{13}C- and ^{31}P NMR spectroscopy (see Fig. 2).

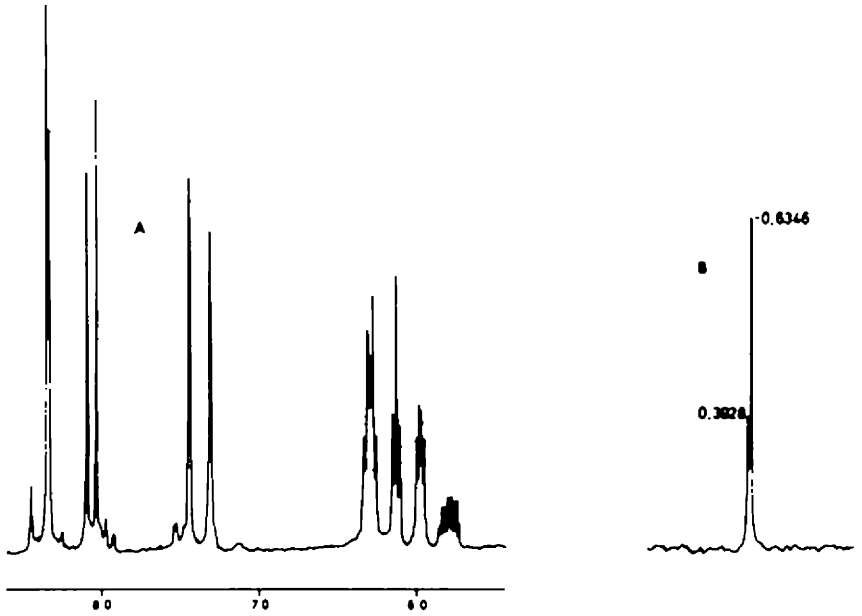


Figure 2. A. Low-field ^1H NMR spectrum of partially-deprotected nucleopeptide H-Ala-Ser(pATAT)-Ala-O-Allyl (12d) showing resonance of: βH of allyl group and H-1' protons of the deoxyriboses (6 ppm region); H-6 protons of thymines (7 ppm region); H-2 and H-8 protons of the adenines (8 ppm region). B. ^{31}P NMR spectrum of 12d showing two resonances in the ratio 1:3.

EXPERIMENTAL

Pyridine, dioxane, tetrahydrofuran (THF) and acetonitrile were dried by refluxing with calcium hydride for 16 h and then distilled. Pyridine was redistilled from p-toluenesulfonyl chloride (50 g/l) and stored over molecular sieves 4Å. Dioxane and THF were redistilled from $LiAlH_4$ (5g/l) and stored over molecular sieves 4Å. N-methylimidazole was distilled from calcium hydride under reduced pressure and stored over molecular sieves 4Å. Dimethylformamide was dried by stirring with calcium hydride overnight at 20°C, distilled under reduced pressure and stored over molecular sieves 4Å. Dichloromethane and methanol were used without further purification. HCl-Tyr-NH_2 was purchased from Novabiochem. 2-Nitrophenylsulfenyl chloride and 1-hydroxy-benzotriazole were purchased from FLUKA. The latter compound was dried over phosphorus pentoxide for 70 h at 50°C. Levulinoic acid anhydride was prepared by

condensing levulinoic acid with DCC.
Triethylammonium bicarbonate buffer (TEAB) was prepared by passing a stream of CO_2 gas through a cooled (ice water bath) solution of triethylamine (825 ml) in water (2175 ml) until a neutral solution was obtained. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC analysis in the solvent systems: A) 8% MeOH in CH_2Cl_2; B) 6% MeOH in CH_2Cl_2; C) 4% MeOH in CH_2Cl_2. Short column chromatography was performed on Kieselgel 60 (230-400 mesh). The column was eluted with CH_2Cl_2 applying a methanol gradient 0-10%. Sephadex LH20 suspended in CH_2Cl_2/MeOH (1/2, v/v, column: 150 cm x 1 cm), and Sephadex G-25 suspended in aqueous NH_4OAc (0.05 M) at pH 6 were also used as chromatography material. FPLC analysis was carried out on a Pharmacia FPLC system using a Mono Q HR (5/5) column. Gradient elution was performed at 20°C, by building up a gradient, starting with buffer A (0.05 M NaH_2PO_4, pH 6) and applying buffer B (0.05 M NaH_2PO_4, 1.2 M NaCl; pH 6) with a flow rate of 2.0 ml/min.
^1H, ^{31}P and ^{13}C NMR spectra were measured at 200 MHz, 20.7 MHz and 50.3 MHz respectively, using a JEOL JNM-FX 200 spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane for ^1H NMR and relative to 85% H_3PO_4 (external standard) for ^{31}P NMR.
^1H NMR spectra were also recorded at 300 MHz using a Bruker WM-300 spectrometer interfaced with an ASPECT-2000 computer. NMR samples were lyophilized three times from D_2O (99.75%) and finally dissolved in D_2O (99.95%). $[\alpha]_D^{20}$ values were measured using a Perkin-Elmer 141 polarimeter.

5'-O-(Dimethoxytrityl)-6-N-(2-nitrophenylsulfenyl)-deoxyadenosine 1
Deoxyadenosine (20.0 mmol, 5.02 g) was coevaporated with acetonitrile and subsequently dissolved in anhydrous acetonitrile (100.0 ml). After addition of hexamethyldisilazane (80.0 mmol, 16.9 ml) and trimethylsilyl chloride (1.0 ml), the mixture was left at 20°C for 1 h. Ammonium chloride was removed by filtration under a stream of nitrogen, and the filtrate was concentrated, and coevaporated with xylene (2 x 50.0 ml). Addition of acetonitrile and pyridine (1/1, v/v, 100.0 ml) to the residue afforded a 0.2 M stock solution of silylated deoxyadenosine. A part of the above obtained stock solution (5.0 mmol, 25.0 ml) was treated with 2-nitrophenylsulfenyl chloride (5.5 mmol, 1.05 g) and the mixture was left for 16 h at 20°C.
The TMS groups were hydrolyzed with methanol/water (1/1, v/v, 5.0 ml). The solution was concentrated and the residue was dissolved in ethyl acetate (100.0 ml), washed with water (2 x 50 ml), concentrated and finally dissolved in dioxane. After refluxing for 20 min, undissolved material was removed by filtration. The solution was concentrated and the residue was coevaporated with pyridine (3 x 30.0 ml), redissolved in pyridine and treated with 4,4'-dimethoxytrityl chloride (6.0 mmol, 2.03 g). After 2 h, the reaction mixture was quenched with methanol (0.5 ml), concentrated, dissolved in CH_2Cl_2 (50.0 ml) and washed with $NaHCO_3$ (1 M, 50.0 ml) and by brine (50.0 ml). The organic phase was dried ($MgSO_4$), concentrated, and the crude residue was purified by short-column chromatography, applying a 0-10% gradient of MeOH in CH_2Cl_2. Yield 2.29 g (3.25 mmol, 65%). Rf 0.72 (A), 0.61 (B), 0.32 (C).
^1H NMR (200 MHz) in $CDCl_3$: δ 8.46 (s, 1H, H-8), 8.34 and 8.30 (d, 1H, J 8.28 Hz, NPS), 8.09 (s, 1H, H-2), 7.44 - 6.77 (m, 13H and 2 x s, 4H, aromatic H of DMT and NPS), 6.46, 6.43 and 6.40 (t, 1H, H-1'), 4.80 - 4.60 (broad, 1H, H-3'), 4.14 - 4.12 (m, 1H, H-4'), 3.76 (s, 6H, OCH_3 of DMT), 3.43 - 3.41 (d, 2H, H-5' and H-5" J 4.87 Hz), 2.93 - 2.54 (2 x m, 2H, H-2' and H-2").
Anal. Calcd. for $C_{37}H_{34}N_6O_7S$: C 62.88, H 4.85, N 11.89; found C 62.78, H 5.30, N 11.33%.

Preparation of dimer 5a
Compound 1 (5.0 mmol, 3.53 g) was dissolved in anhydrous pyridine (25.0 ml) and coevaporated to dryness. O-(2-chlorophenyl)-O,O-bis(1-benzotriazolyl)phosphate 0.2 M solution in dioxane (5.75 mmol, 28.8 ml) was added and the mixture was stirred for 20 min at 20°C. TLC analysis (system A) showed that monomer 1 was completely converted to intermediate 3 (R_f 0). Thymidine (6.25 mmol, 1.51 g) in anhydrous pyridine (30.0 ml) was added to intermediate 3. After stirring for 2 h at 20°C, TLC analysis (systems A,B and C) indicated the absence of 3. The mixture was diluted with CH_2Cl_2 (300.0 ml) and subsequently washed with TEAB (1M, 100.0 ml) and water (100.0 ml). The organic layer was dried ($MgSO_4$) and concentrated to give an oil. The crude dimer was triturated with petroleum-ether (40-60°C) and purified by column-chromatography (silica gel in CH_2Cl_2), applying a 0-10% gradient of MeOH in CH_2Cl_2. Yield 3.92 g (3.50 mmol, 70%). Rf 0.57 (A), 0.38 (B), 0.17 (C). ^{31}P NMR in CH_2Cl_2: δ -7.43 and -8.34.

Synthesis of dimer 5c
Levulinoic acid anhydride in dioxane (1 M, 1.5 mmol, 1.5 ml) and N-methylimidazole (0.5 mmol, 0.04 ml) were added to dimer 5a (1.0 mmol, 1.12 g) in anhydrous pyridine (1.0 ml) at 0°C. After stirring for 2 h, TLC analysis (systems A, B and C) indicated conversion of 5a into 5b. The mixture was diluted with CH_2Cl_2 (50.0 ml) and extracted with TEAB (1 M, 50.0 ml) and with water (50.0 ml). The solution was dried ($MgSO_4$), concentrated and dissolved in a minimal amount of CH_2Cl_2/MeOH (7/3, v/v) to which p-toluenesulfonic acid (2%) in CH_2Cl_2/MeOH (7/3, v/v, 12.0 ml) was added at 0°C. When TLC analysis indicated complete detritylation, the reaction was quenched with aqueous $NaHCO_3$ (1 M, 5.0 ml), diluted with CH_2Cl_2 (50.0 ml) and extracted with $NaHCO_3$ (25.0 ml). The organic layer was dried ($MgSO_4$), concentrated and the crude residue was purified by short column chromatography. Yield 0.83 g (0.9 mmol, 90%). Rf 0.63 (A), 0.52 (B), 0.22 (C).
^{31}P NMR in CH_2Cl_2/MeOH: δ -7.13, -7.22 and -7.68.

Synthesis of tetramer 6b
Dimer 5a (1.44 mmol, 1.62 g) was dissolved in anhydrous pyridine and coevaporated to dryness. Phosphorylating agent 2 (1.74 mmol, 8.7 ml) was added, and after 20 min, TLC analysis (system A), showed complete conversion of 5a into 5d. Introduction of dimer 5c (1.8 mmol,

1.65 g) and N-methylimidazole (6.0 mmol, 0.48 ml) followed by stirring at 20°C for 2 h yielded crude tetramer 6a. After standard work up procedure detritylation and purification as described for dimer 5c, pure tetramer 6b was obtained. Yield 2.36 g (1.24 mmol, 86%), Rf 0.56 (A), 0.36 (B), 0.11 (C).
^{31}P NMR in CH_2Cl_2: δ -7.07, -7.25, -7.77 and -7.89.

Synthesis of dipeptide 7a

To a solution of NPS-Phe-OH (5.0 mmol, 1.6 g) in DMF (25.0 ml), HCl-Tyr-NH_2 (5.0 mmol, 1.1 g), triethylamine (5.0 mmol, 0.7 ml) and hydroxybenzotriazole (6.0 mmol, 0.81 g) were added. The solution was cooled to 0°C and dicyclohexylcarbodiimide (5.5 mmol 1.13 g) was added. After stirring at 0°C for 1 h, the cooling was removed and the mixture was left 18 h at 20°C. Dicyclohexylurea was removed by filtration over a small layer of celite and the filtrate was concentrated. The residue was diluted with CH_2Cl_2 (100.0 ml) and extracted with aqueous KH_2PO_4 (1 M, 2 x 25.0 ml, pH 3.8), water (25.0 ml), aqueous Na_2CO_3 (1 M, 2 x 25.0 ml) and finally with water (25.0 ml). The organic phase was dried ($MgSO_4$), and subsequently concentrated to dryness. Crystallization of the residue from ethyl acetate afforded pure dipeptide 7a. Yield: 1.92 g (4.0 mmol, 80%), R_f 0.54 (A), 0.31 (B), 0.17 (C), $[\alpha]_D^{20}$ +5.8 (c 1, MeOH), mp. 119-121°C. ^{1}H NMR (200 MHz) in DMSO/$CDCl_3$: δ 8.12 - 6.68 (13H, aromatic), 4.70 and 4.65 (broad d, 1H, J 8.53 Hz, CH), 4.39 and 4.34 (d, 1H, J 8.53 Hz, CH), 3.01 (m, 4H, CH_2 of Tyr and Phe).
^{13}C NMR in DMSO/$CDCl_3$: δ 172.48 and 171.90 (2xC=O of Tyr and Phe), 154.91 - 114.17 (aromatic C of NPS, Tyr and Phe), 53.12 and 51.54 (αCH of Tyr and Phe), 38.75 and 35.96 (βCH_2 of Tyr and Phe).

Fully protected nucleopeptide 8a

The synthesis of 8a was performed in a similar fashion as described for the synthesis of tetramer 6b starting from dipeptide 7a (0.5 mmol, 0.24 g) and reagent 2 (0.6 mmol, 3.0 ml) in anhydrous dioxane, followed by the addition of tetramer 6b (0.625 mmol, 1.19 g) in the presence of N-methylimidazole (2.08 mmol, 0.17 ml). Yield of pure 8a was 1.12 g (0.44 mmol, 88%), R_f 0.55 (A), 0.32 (B), 0.07 (C).
^{31}P NMR in CH_2Cl_2: δ -6.47, -6.92, -7.22, -11.85, and -12.06.

Partially deblocked nucleopeptide 8b

The removal of the 2-ClC_6H_4O group was performed following two procedures:
a) Nucleopeptide 8a (0.04 mmol, 102 mg) was treated with syn-4-nitrobenzaldoxime (1.6 mmol, 0.266 g) and N^1,N^1,N^3,N^3-tetramethylguanidine (1.28 mmol, 0.147 g) in anhydrous THF (4.0 ml). ^{31}P NMR spectroscopy revealed complete conversion of 8a to 8b within 18 h at 20°C. The reaction was quenched by adding acetic acid (1.58 mmol, 0.09 ml) and the solvent was removed under reduced pressure. The residue was diluted with CH_2Cl_2/MeOH 1/2, v/v and applied to a Sephadex LH-20 column which was eluted with the same eluent.
^{31}P NMR in THF: δ -1.30, -1.51, -1.60, -5.68 and -5.77.
b) Nucleopeptide 8a (0.02 mmol, 51.2 mg) was dissolved in a solution of tetrabutylammonium fluoride in pyridine/water (1/1, v/v, 0.25 M, 3.2 ml) and the mixture was stirred for 24 h at 20°C. Removal of 2-ClC_6H_4O from 8a was monitored by ^{31}P NMR. Crude 8b thus obtained was purified as described under a).
^{31}P NMR in pyridine/water: δ -0.24, -0.44, -0.59 and -4.43.

Partially deblocked nucleopeptide 8c

Compound 8b obtained above was dissolved in a minimum amount of dioxane/water (9/1, v/v) and treated with tri-n-butylphosphine (5.0 eq). After 20 min at 20°C, TLC analysis indicated complete conversion of 8b to 8c (system A). After removal of the solvent, the residue was dissolved in CH_2Cl_2/MeOH (30 ml, 1:2 v/v), and applied to a Sephadex LH-20 column which was eluted with the same solvent.

Completely deblocked nucleopeptide 8d

A solution of hydrazine hydrate in pyridine/acetic acid (3/2, v/v, 0.5 M, 5.0 eq) was added to nucleopeptide 8c. After 20 min, the reaction was quenched with acetylacetone (0.97 mmol, 0.1 ml) and crude 8d was applied to a Sephadex G-25 column. The fractions, containing fully deblocked nucleopeptide 8d were concentrated and applied to a column of Dowex 50W cation exchange resin (Na^+ form). The UV-positive fractions were collected, pooled, concentrated to small volume and finally lyophilized. Yield of pure 8d was 39.6 mg (0.023 mmol, 58%) by using oximate-ions assisted deblocking of 8a, and 14.0 mg (0.008 mmol, 41%), by using fluoride ions for the removal of 2-chlorophenyl groups from 8a, $[\alpha]_D^{20}$ +38.7 (c 1, H_2O), retention time (FPLC-analysis) 5.2 min.
Anal. Calcd. for $C_{60}H_{71}N_{17}O_{29}P_4Na_4$: P 7.24; found P 7.10%.
^{1}H NMR (300 MHz) in D_2O: δ 8.33 and 8.11 (2xs, 2H, 2xH-8 dA), 8.03 and 8.01 (2xs, 2H, 2xH-2 dA), 7.44 and 7.28 (2xs, 2H, 2xH-6 dT), 7.23 and 7.21 (d, 2H, J 7.38 Hz, Tyr), 7.05 and 7.03 (d, 2H, J 6.61 Hz, Tyr), 6.83 and 6.82 (5H, Phe), 6.29 - 5.95 (3xt, 4H, 4xH-1', J 6.8, 7.2, 6.8, 5.8 and 5.6 Hz), 4.96 - 3.58 (4xH-3', 4xH-4', 2x α H, 4xH-5' and 4xH-5"), 2.97 - 2.12 (12H, 4xH-2', 4xH-2" and 2x βCH_2 dd at 2.97 - 2.95 J 6.3 Hz and 2.93 - 2.91 J 5.9 Hz), 1.69 and 1.66 (2xs, 6H, 2xCH_3 dT).
^{13}C NMR D_2O: δ 177.36 and 176.17 (C=O of Tyr and Phe), 157.07 - 112.48 (C-4, C-2, C-6, C-5 of dT, C-6, C-2, C-4, C-8, C-5 of dA and aromatic C of Tyr and Phe), 86.08 - 84.22 (C-4' and C-1' of dA and dT), 77.44 - 65.67 (C-3' and C-5' of dA and dT), 57.03 and 55.10 (αCH of Tyr and Phe), 41.29 - 37.23 (C-2' of dA and dT and βCH_2 of Tyr and Phe), 13.79 and 13.29 (2xCH_3 dT).
^{31}P NMR in D_2O: δ -0.60, -0.66 and -3.96.

Synthesis of tripeptide 9

To a solution of H-Ser-OH (7.5 mmol, 0.79 g) in dimethoxyethane (10.0 mmol) and aqueous

$NaHCO_3$ (1 M, 10.0 mmol, 10.0 ml), NPS-Ala-OSu (5.0 mmol, 1.70 g) was added in five portions over a period of 30 min. After 24 h at 20°C, the dimethoxyethane was removed under reduced pressure. Addition of water (50.0 ml) and ethyl acetate (100.0 ml) followed by acidification (1 N H_2SO_4, pH 3), afforded, after removal of the aqueous layer, a solution of the crude dipeptide in ethyl acetate. Subsequently, the water layer was washed twice with ethyl acetate (2 x 20 ml), and the combined organic layers were washed with brine (100.0 ml). The yellow organic phase was dried ($MgSO_4$), concentrated to small volume and diluted with petroleum ether. Yield of the crystalline dipeptide was 1.32 g (4.0 mmol, 80%), R_f 0.27 (CH_2Cl_2/MeOH/AcOH, 85/10/5, v/v/v), mp. 112-115°C, Σ of NPS-Ala-Ser-OH (ref. Glu) 0.93, pH 4.88.

To a solution of dipeptide NPS-Ala-Ser-OH (3.0 mmol, 0.99 g) in THF (50.0 ml) N-methylmorpholine (3.2 mmol, 0.35 ml) was added and the mixture was cooled to -15°C. Isobutyl chloroformate[21] (3.15 mmol, 0.42 ml) was added, and the mixture was left for 10 min at -15°C. p-Toluenesulfonic acid salt of H-Ala-OAllyl (3.0 mmol, 0.9 g) and N-methylmorpholine (3.0 mmol, 0.33 ml) were now added. The mixture was stirred for 15 min at -15°C and subsequently for 1 h at 20°C. The solution was concentrated, the residue was diluted with CH_2Cl_2 (100.0 ml) containing MeOH (2.0 ml) and extracted with aqueous Na_2CO_3 (1M, 2x50.0 ml), water (50.0 ml) and finally KH_2PO_4 buffer (1M, 2x50.0 ml, pH 3.8). The organic layer was washed with water (50.0 ml), dried ($MgSO_4$), concentrated and the tripeptide was crystallized from EtOAc/CH_2Cl_2/hexane. Yield of 9 was 1.15 g (2.61 mmol, 87%), Rf 0.65 (A), mp. 116-118°C, $[\alpha]_D^{20}$ -59.2 (c 1, dioxane). ^1H NMR (200 MHz) in DMSO: δ 8.29-7.32 (4H, aromatic NPS), 5.94-5.77 (m, 1H, CH=, Allyl), 5.32-5.08 (m, 2H, CH_2=, Allyl), 4.89-3.55 (7H, 3xαCH, βCH$_2$ Ser, and CH_2 Allyl), 1.31-1.28 (d, 6H, 2xCH_3, Ala).
^{13}C NMR in DMSO: δ 173.53, 171.95 and 169.76 (3xC=O of 2xAla and Ser), 145.61-124.79 (aromatic C), 134.28 (CH=, Allyl), 117.61 (CH_2=, Allyl), 64.82 and 61.72 (CH_2, Allyl and βCH$_2$, Ser), 58.86, 54.86 and 47.68 (3xαCH, of 2xAla and Ser), 19.21 and 17.08 (2xβCH$_3$, Ala).

Fully protected nucleopeptide 12f

Tripeptide 9 (0.227 mmol, 99.7 mg) in anhydrous acetonitrile (3.0 ml) containing 1-H-tetrazole (0.227 mmol) was phosphorylated with allyl-bis(diisopropylamino)phosphine in acetonitrile (1.25 M, 0.18 ml). TLC analysis (systems A and B), after 30 min, indicated complete conversion of compound 9 to intermediate 11. Subsequently, tetramer 6b (0.227 mmol, 433 mg) and 1H-tetrazole (0.227 mmol) were added and the mixture was left for 18 h at 20°C. Oxidation at 0°C for 30 min. with t-butyl hydroperoxide (4.0 mmol, 0.5 ml, 80% solution in di-tert-butylperoxide), afforded crude nucleopeptide 12f. The solution was diluted with CH_2Cl_2 (50.0 ml), washed with water (30.0 ml), dried ($MgSO_4$) and concentrated. The oily residue was then dissolved in CH_2Cl_2/MeOH (1/2 v/v) and applied to a Sephadex LH-20 column which was eluted with the same solvent. Further purification was obtained by short column chromatography. Yield of 12 was 500 mg (0.204 mmol, 90%), Rf 0.63 and 0.48 (A). ^{31}P NMR in CH_2Cl_2: δ -0.66, -7.31 and -7.43.

Fully protected nucleopeptide 12a

Tetramer 6b (0.227 mmol, 433 mg) was dissolved in anhydrous pyridine and coevaporated to dryness. Phosphorylating reagent 2 (0.261 mmol, 1.31 ml) was added and TLC analysis (system A) after 20 min showed complete conversion of 6b into intermediate 6c. Tripeptide 9 (0.283 mmol, 124 mg) in anhydrous pyridine (1.5 ml), which was previously dried by coevaporation with pyridine (2x3.0 ml), was added to intermediate 6b. After stirring for 2h at 20°C, TLC analysis (CH_2Cl_2/MeOH 9/1 v/v) indicated the absence of compound 6c. Work up and purification procedures were performed in a similar fashion as described for compound 12f. Yield of 12a was 366 mg (0.145 mmol, 64%), Rf 0.46 (CH_2Cl_2/MeOH, 9/1, v/v). ^{31}P NMR in CH_2Cl_2: δ -6.89,-7.28 and -7.43.

Partially deblocked nucleopeptide 12b

Fully protected heptamer 12a (0.04 mmol, 100 mg) was treated with tetrabutylammonium fluoride in a similar way as described for the deblocking of nucleopeptide 8a (method b). ^{31}P NMR in pyridine/water: δ 0.97, -0.24, -0.48 and -0.57.

Partially deblocked nucleopeptide 12g

Nucleopeptide 12f (0.02 mmol, 50 mg) was dissolved in pyridine/water (1/1 v/v, 2.5 ml) and was left at 20°C for 48 h, after which time ^{31}P NMR showed the reaction to be complete (pyridine/water: δ -0.91, -7.10 and -7.40). To a solution of 12g thus obtained in pyridine/H_2O tetrabutylammonium fluoride was added to afford partially deblocked nucleopeptide 12b.

Partially deblocked nucleopeptide 12c

Nucleopeptide 12b was treated with tri-n-butylphosphine in a similar fashion as described above for the synthesis of compound 8c.

Partially deblocked nucleopeptide 12d

Hydrazinolysis of the levulinoyl group and purification of crude compound 12d were accomplished in a similar manner as described above for the synthesis of completely deblocked nucleopeptide 8d. Yield of 12d was 23.4 mg (0.014 mmol, 70%). $[\alpha]_D^{20}$ -10.1 (c 1, dioxane/water, 1/1, v/v), retention time (FPLC-analysis) 3.2 min, Anal. Calcd. for $C_{54}H_{71}N_{17}O_{31}P_4Na_4$: P 7.42; found P 7.39%. ^1H NMR (300 MHz) in D_2O: δ 8.34 and 8.33 (2xs, 2H, 2xH-8, dA), 8.10 and 8.05 (2xs, 2H, 2xH-2, dA), 7.46 and 7.31 (2xs, 2H, 2xH-6, dT), 6.35-5.98 (4H, 4xH-1'), 5.85-5.76 (m, 1H, CH=, Allyl), 5.23-5.16 (m, 2H, CH_2=, Allyl), 4.97-3.51 (23H, 4xH-3', 4xH-4', 3xαCH, βCH$_2$ Ser, CH_2 Allyl, 4xH-5' and 4xH-5''), 2.81-1.91 (8H, 4xH-2' and 4xH-2''), 1.69 (6H, 2xCH_3, dT), 1.35-1.24 (6H, 2xβCH3, Ala).
^{13}C NMR in D_2O/TMA: δ 174.55, 171.89 and 170.96 (C=O, of 2xAla and Ser), 166.49 - 112.24 (C-

4, C-2, C-6, C-5 of dT and C-6, C-2, C-4, C-8, C-5 of dA), 134.43 (CH= allyl), 119.27 (CH_2= allyl), 85.75 - 84.61 (C-4' and C-1' of dA and dT), 77.19 - 70.71 (C-3' of dA and dT). 67.33 - 60.78 (C-5' of dA and dT, βCH_2 Ser and CH_2 allyl), 55.03, 54.19 and 49.95 (3xαCH, of 2xAla and Ser), 44.49 - 39.26 (C-2' of dA and dT), 17.48 and 16.99 (2xβCH_3 Ala), 13.83 and 12.55 (2 x CH_3 dT).
^{31}P NMR in D_2O: δ 0.39 and -0.63.

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