Synthesis of Nucleopeptides by Employing an Enzyme-Labile Urethane Protecting Group

Duraiswamy A. Jeyaraj,[a, b] Heino Prinz,[a] and Herbert Waldmann*[a, b]

Abstract: Nucleoproteins are naturally occurring biopolymers in which the hydroxy group of a serine, a threonine, or a tyrosine moiety is linked through a phosphodiester group to the 3′- or 5′-end of a nucleic acid. For the study of the biological phenomena in which nucleoproteins are involved, for example, viral replication, nucleopeptides embodying the characteristic linkage between the peptide chain and the oligonucleotide may serve as powerful tools. However, as a result of the multifunctionality and the pronounced acid and base lability of nucleopeptides, their synthesis requires the application of a variety of orthogonally stable blocking groups, which can be removed under the mildest conditions. We have developed a new mild enzymatic deprotection method, that is, the penicillin G acylase-catalyzed hydrolysis of the *N*-phenylacetoxybenzyloxycarbony (PhAcOZ) group, for the synthesis of nucleopeptides. We demonstrate the wide applicability of this method by coupling the *N*-terminally deprotected nucleopeptides **31a–c** with PhAcOZ-protected amino acids and subsequent removal of the *N*-PhAcOZ group from fully protected nucleotetrapeptides **32a**,**b** with penicillin G acylase. The reaction conditions are very mild (pH 6.8) so that no undesired side reaction such as cleavage of the nucleotide bond or β-elimination of the nucleotide was observed.

Keywords: enzymes · nucleopeptides · penicillin G acylase · protecting groups

Introduction

Nucleoproteins are naturally occurring biopolymers in which the hydroxy group of a serine, a threonine, or a tyrosine moiety is linked through a phosphodiester group to the 3′- or 5′-end of a nucleic acid.[1] These protein conjugates play decisive roles in important biological processes such as viral replication.[2] The emerging biological importance of nucleoproteins was recently highlighted by the finding that the p53 protein is a nucleoprotein.[3] Nucleoprotein p53 is a tumour suppressor protein that is critically involved in cell-cycle regulation.[4] It can potently suppress cellular transformation, inhibit tumorigenesis, arrest cell growth, and induce apoptotic death. In its biologically active form, p53 is covalently linked at Ser-386 to a nucleic acid through a 5′-phosphodiester group[5] and the serine is flanked by two aspartic acid residues (Figure 1).

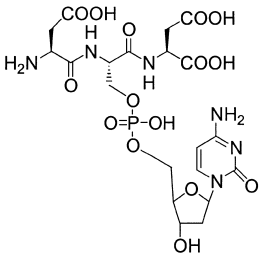

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Figure 1. Structure of the nucleopeptide representing the linking region between peptide and nucleotide in the p53 protein.

For the study of the biological phenomena in which nucleoproteins are involved, nucleopeptides embodying the characteristic linkage between the peptide chain and the oligonucleotide may serve as powerful tools. However, as a result of the multifunctionality of nucleopeptides their synthesis requires the application of a variety of orthogonally stable blocking groups. For example, different functionalities such as hydroxyl, amine, phosphate, acid, and amine groups require the application of different types of protecting groups that can be selectively cleaved under very mild conditions. In addition, fully protected serine/threonine-containing nucleopeptides are very acid and base labile; this makes most of the established blocking functions inapplicable to nucleopeptide synthesis. Under acidic conditions the purine nucleotide may be depurinated[6] and under basic conditions the entire oligonucleotide part may be split off by β-elimination (Figure 2).

Thus, it is not surprising that only a few reports on the successful synthesis of nucleopeptides have appeared.[7–10] In

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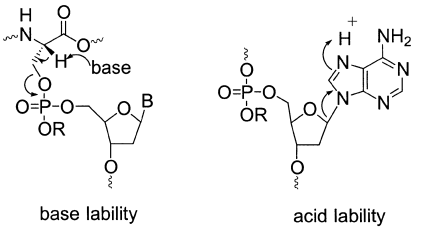


Figure 2. Acid and base lability of nucleopeptides. B = adenine, cytosine, guanine, or thymine.

particular, the development of protecting groups allowing the assembly of sensitive serine/threonine-containing nucleopeptides through sequential selective *N*-terminal deprotection and chain elongation has posed a major problem. However, the application of enzyme protecting-group techniques may offer an alternative to classical chemical methods.[11] In this paper we report that the enzyme-labile phenylacetoxy benzyloxycarbonyl group (PhAcOZ)[12] fulfils the extraordinary demands of nucleopeptide chemistry concerning chemoselectivity and mildness of cleavage conditions.[13] The PhAcOZ group is an enzyme-labile urethane that is cleaved under very mild conditions by penicillin G acylase (PGA). PGA hydrolyzes the phenylacetic acid phenyl ester, and subsequent fragmentation of the resultant phenolate gives a quinone methide (which is trapped by nucleophiles) and the desired unmasked amino acid ester (Figure 3).

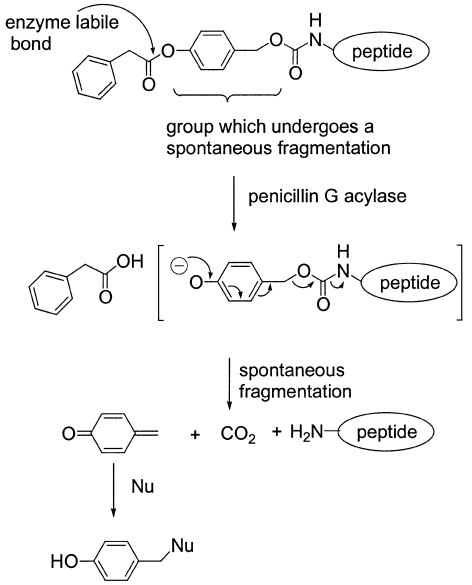
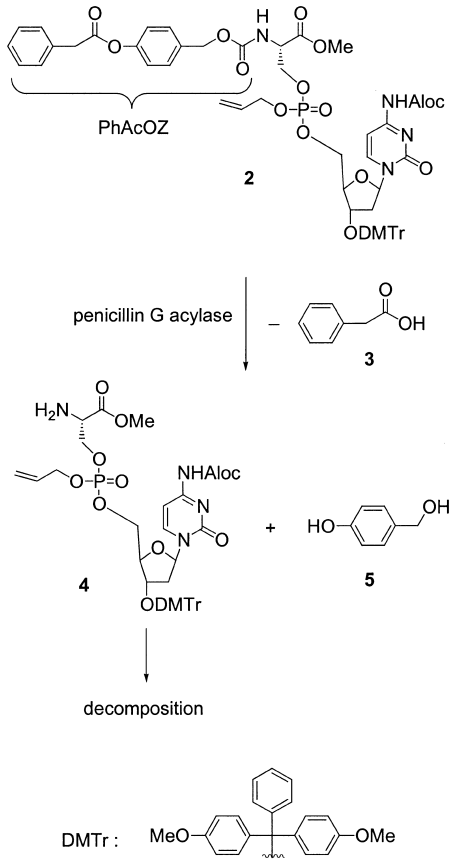


Figure 3. Principle of the enzyme-initiated cleavage of the *N*-phenylacetoxybenzyloxycarbony (PhAcOZ) urethane.

Results and Discussion

In the quest for an enzymatic technique, we focussed on the establishment of a general method for the synthesis of various nucleopeptides under appropriate reaction conditions. We also aimed to synthesize the p53-related nucleopeptide **1** as a particularly demanding example. Thus, we anticipated the need to selectively mask and unmask the side chain carboxylic acids of the aspartic acid (Asp) residues and the pronounced tendency of Asp to undergo undesired side reactions (see below) would significantly complicate the matter.

In initial experiments aimed at the enzymatic deprotection of nucleopeptides, *N*-PhAcOZ-protected serine nucleotide esters like **2** were treated with penicillin G acylase (PGA). In the ensuing enzymatic reaction the urethane group was indeed cleaved (Scheme 1).



Scheme 1. Penicillin G acylase-mediated cleavage of *N*-PhAcOZ-protected serine nucleotide **2**.

However, the *N*-terminally unmasked nucleoamino acid ester **4** could not be isolated. Rather, the reaction product decomposed under the reaction conditions, probably through elimination of the nucleotide.

To overcome this problem in a second series of experiments, dipeptide – nucleotide esters were subjected to the enzymatic reaction conditions. As hoped, the deprotection occurred smoothly and rapidly, but the liberated dinucleopeptide esters underwent competing diketopiperazine formation, a well-known and undesired side reaction in peptide chemistry.

Therefore, nucleotripeptides were chosen as an appropriate starting point to investigate the applicability of the PhAcOZ group in nucleopeptide chemistry.

Initial experiments were carried out by employing an aspartyl nucleopeptide corresponding to **1** as the substrate in the enzymatic deprotection reaction. To this end, tripeptide **15** was prepared as summarized in Scheme 2. The fully protected

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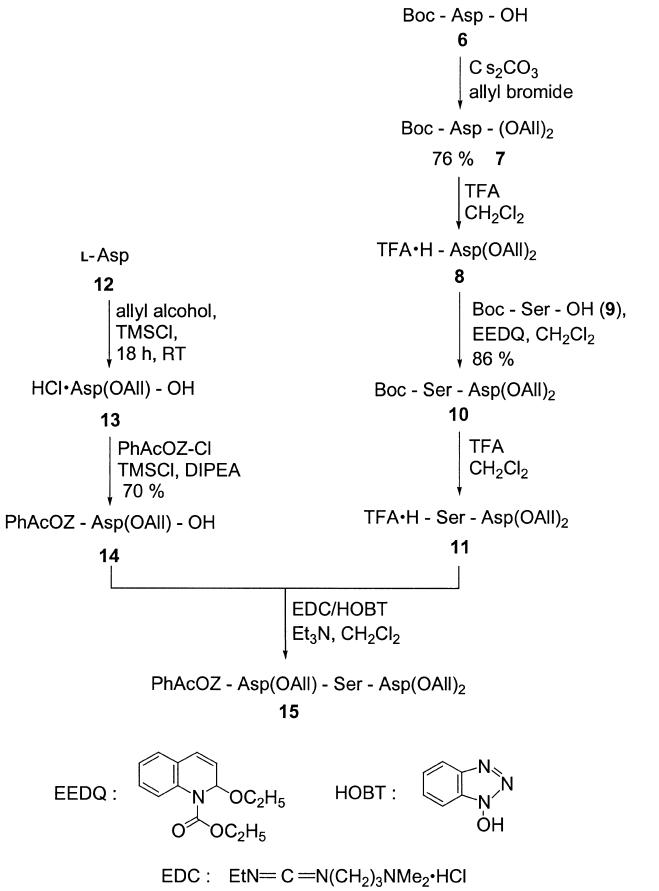
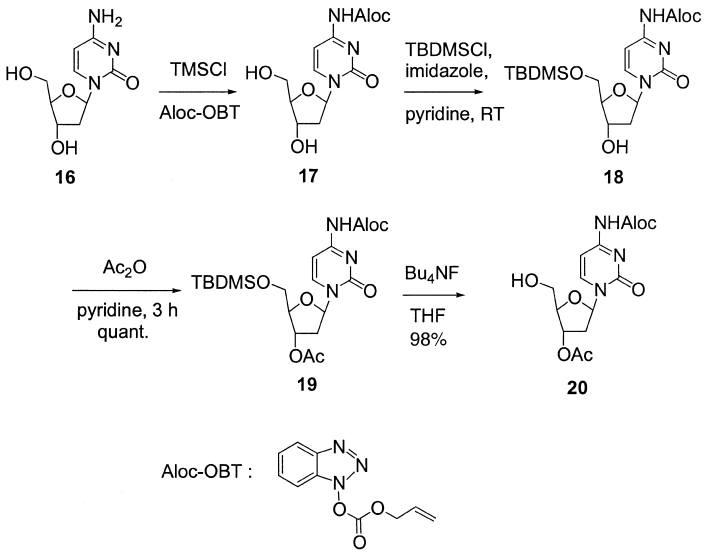


Scheme 2. Synthesis of *N*-PhAcOZ-protected tripeptide **15**.

aspartic acid **7** was obtained in high yield from the cesium salt of Boc-Asp-OH by treatment with allyl bromide.[14] Then, the Boc group was cleaved from **7** with trifluoroacetic acid (TFA) to give the aspartic acid diallyl ester TFA salt **8** in quantitative yield. Aspartic acid diallyl ester **8** was coupled with Boc-Ser-OH (**9**) in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), to furnish dipeptide **10** in high yield. The Boc group was cleaved with TFA in dichloromethane to give the dipeptide TFA salt **11**. To synthesize the PhAcOZ-protected aspartic acid derivative **14**, the γ-carboxylic acid of aspartic acid **12** was selectively esterified according to a known procedure.[15] Treatment of **13** with PhAcOZ-Cl then gave the aspartic acid derivative **14** in high yield. Coupling of compounds **14** and **11** in the presence of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC)/1-hydroxybenzotriazole (HOBT) and triethylamine proceeded smoothly to yield tripeptide **15**.

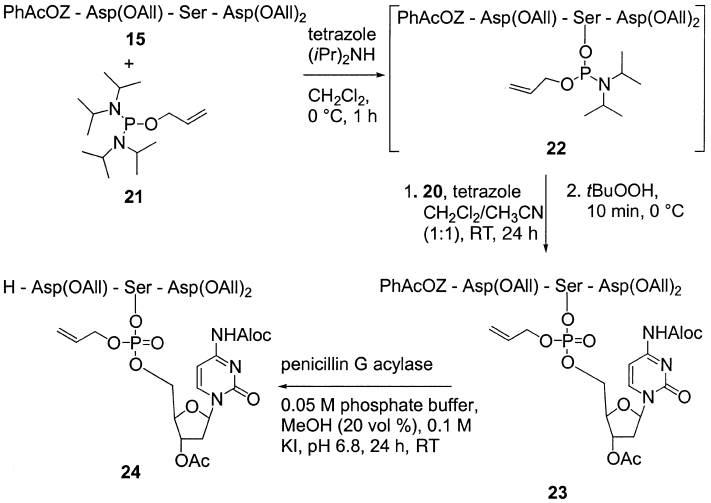
The required partially protected deoxycytidine was prepared as shown in Scheme 3. The free amino group present in deoxycytidine **16** was protected with an allyloxycarbonyl (Aloc) protecting group by reaction with allyloxycarbonyloxybenzotriazole (Aloc-OBT) following a literature procedure.[16] Then, the 5′-OH group of **17** was selectively masked as a tetrabutyldimethylsilyl (TBDMS) ether by reaction with one equivalent of TBDMSCl.[17] Compound **18** was acylated at the 3′-OH group by means of acetic anhydride in pyridine to yield the fully protected deoxycytidine **19** in quantitative yield. Selective removal of the TBDMS ether by treatment



Scheme 3. Synthesis of 3′-acetyl-4-*N*-allyloxycarbonyl-2′-deoxycytidine (**20**).

with tetrabutylammonium fluoride (TBAF) gave the desired partially protected deoxycytidine **20** in very high yield.

The central serine of tripeptide **15** was then coupled to the selectively masked deoxycytidine **20** by formation of a phosphodiester bond. Upon treatment of **15** with phosphordiamidite **21**[18] in the presence of tetrazole and diisopropylamine at 0 °C, a hydrolysis-sensitive intermediate **22** was formed. This was converted without isolation into the desired peptide conjugate **23** by treatment with deoxycytidine building block **20** in the presence of tetrazole, and subsequent oxidation of the formed phosphites to the corresponding phosphates by means of *tert*-butyl hydroperoxide at 0 °C (Scheme 4). The product was purified by gel permeation



Scheme 4. Enzymatic cleavage of the PhAcOZ group from nucleotripeptide **23** with penicillin G acylase.

chromatography after filtering through silica gel to yield the nucleotripeptide **23** in 40 % yield.

Enzymatic cleavage of the PhAcOZ group by penicillin G acylase (PGA): Completely masked nucleotripeptide **23** was subjected to selective enzymatic deprotection with PGA. The

PhAcOZ protected nucleopeptide **23** was treated with the enzyme in a mixture of 0.05 M phosphate buffer and methanol (20 vol%) at pH 6.8 and room temperature, in the presence of 0.1 M KI as a trapping reagent for the quinone methide formed in the enzyme-initiated cleavage of the urethane.[12] NMR spectroscopic investigation of the product mixture initially revealed that the aromatic signals characteristic for the PhAcOZ group had disappeared, indicating that the conversion had reached at least 80%. However, unexpectedly, the isolation of the desired nucleopeptide from the reaction mixture proved to be very difficult and yielded only 10–15% of the product. In addition, a major product was formed, which could not be separated readily by means of conventional techniques. These findings suggested either that the enzymatic reaction had not proceeded in the expected way or that an undesired side reaction had occurred once more.

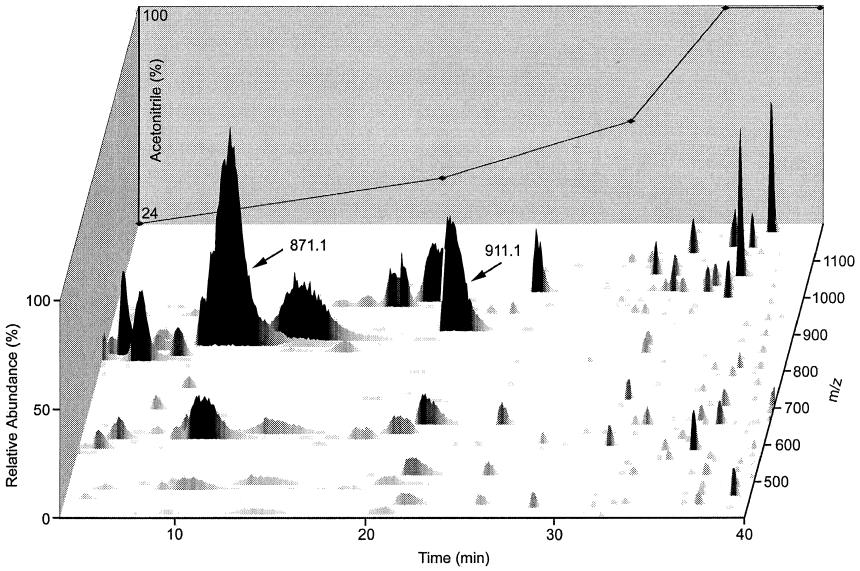


Figure 4. Three-dimensional representation of an HPLC-MS experiment (time vs. m/z vs. relative abundance). The gradient (percentage acetonitrile) is given in the background. The reaction products after the enzymatic deprotection were loaded onto a C18-HD reverse-phase column and eluted with increasing amounts of acetonitrile in 0.1% HCOOH. Masses ranging from m/z 400–1200 are given in the time range from 4–40 minutes.

Numerous attempts were made to overcome this severe problem, for example, by varying different reaction parameters (temperature, pH, co-solvent) and increasing the amount of biocatalyst. However, an increase in yield could not be achieved. In particular, progress was hampered by the fact that conventional analysis and separation techniques did not give access to pure product, or allow clear conclusions to be drawn about the product spectrum formed. After substantial experimentation, this problem was finally fully analyzed by employing mass-spectrometric techniques. Since these techniques subsequently proved to be of major importance in general, they are described below in detail for the example discussed above.

Identification of the reaction products: Identification of the products from the deprotection reaction comprised the main obstacle in the nucleopeptide synthesis. Matrix assisted laser desorption ionization (MALDI) mass spectrometry gave a variety of molecular masses, the relative abundance of which varied with the matrix employed. The mass of the desired product **24** was calculated to be 911 and could not be identified above background level. FAB mass spectrometry likewise allowed the detection of masses ranging from 800–1000, but not of the desired product. Nano-electrospray mass spectrometry indicated that indeed a mass of 911 was present, but its relative abundance was lower than a peak at m/z 909, and thus it might have been an isotope of the latter. Only the coupling of reverse phase chromatography to ESI mass spectrometry (HPLC-MS) allowed the unambiguous identification of the reaction products (Figure 4).

The major product provides a peak at m/z 871, that is 40 mass units less than the expected product of m/z 911. This was in accordance with the NMR spectrum of the crude reaction mixture, which had a major compound with only four allyl groups instead of five allyl groups expected for the desired peptide. We speculated that this peak in the mass spectrum was due to the formation of an intermediate aminosuccinyl derivative **25** (Figure 5).

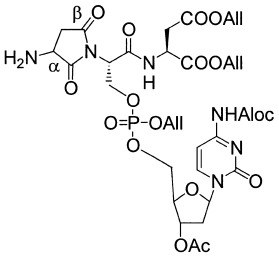


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Figure 5. Possible aminosuccinyl intermediate **25** formed during the reaction.

It has been suggested[19] that aminosuccinyl derivative formation in aqueous solution is a common problem with aspartic acid. Thus aspartyl peptides, in particular sequences that contain aspartylglycine (Asp-Gly) and aspartylserine (Asp-Ser),[20, 21] are prone to cyclize to five-ring imides under acidic or basic conditions.

Fragmentation of the ion at m/z 911 in the mass spectrometer (MS/MS experiments) yielded fragments of m/z 518 and 438, which corresponded to the peptide cleaved at the phosphate with and without HPO_3, respectively. The desired product **24**, was thus identified by its molecular mass (911.1) and from its fragmentation pattern.

Since the compound in the salt form was partially soluble in water, we encountered difficulties in isolating the product from the buffer and the nucleophile used during the enzymatic deprotection. Conventional separation on silica gel and extraction was not effective. Instead, the immobilized enzyme was filtered and the solution was lyophilized. The phosphate salts and the KI were separated from the required compound

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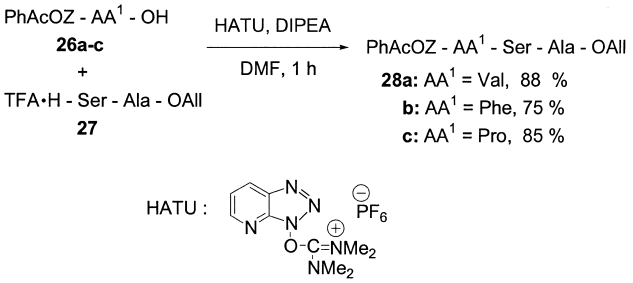
by filtering through a C18 Sep-Pak cartridge and washing with water (2 mL). The compound adsorbed on the C18 cartridge was then eluted with methanol. The compound isolated by this method was almost pure.

These results clearly demonstrated that the enzymatic protecting group technique had, in principle, worked well. However, once more, an amino-acid-dependent side reaction that was independent of the newly investigated blocking group prevented a clear demonstration of the suitability of the PhAcOZ group for nucleopeptide synthesis being achieved.

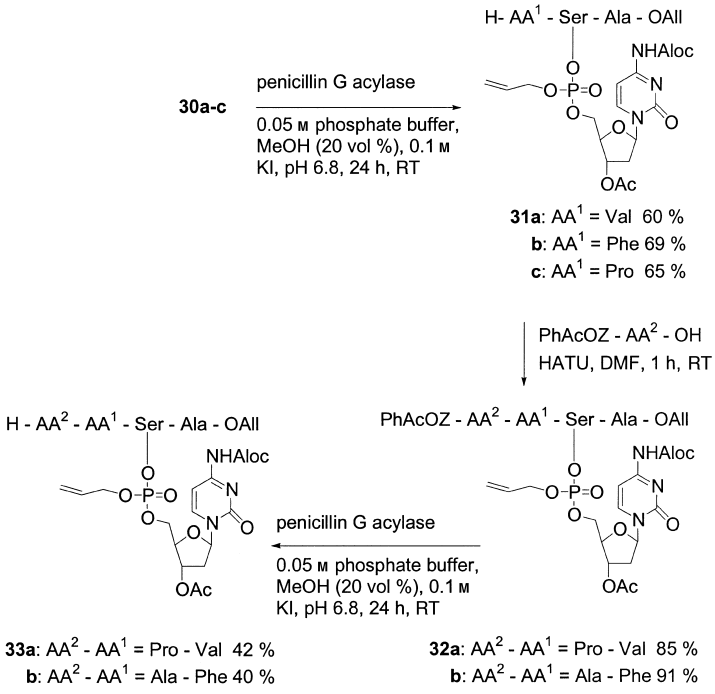
To determine if the conditions for the selective removal of this enzyme-sensitive blocking group were mild enough to allow an efficient nucleopeptide synthesis, different model nucleotripeptides were synthesized. PhAcOZ-AA^1-OH were prepared from the corresponding amino acids and PhAcOZ-Cl by a known procedure in high yields.[12] The PhAcOZ-masked amino acids **26a–c** and H-Ser-AlaOAll (**27**), prepared by literature procedures,[22] were coupled in the presence of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine in DMF at 0 °C for one hour to yield the PhAcOZ-protected tripeptides **28a–c** in very high yield (Scheme 5). In the presence of EDC/HOBT, the coupling was not efficient and the tripeptides were isolated in only 30–40% yield.



Scheme 5. Synthesis of PhAcOZ protected tripeptides **28a–c**.

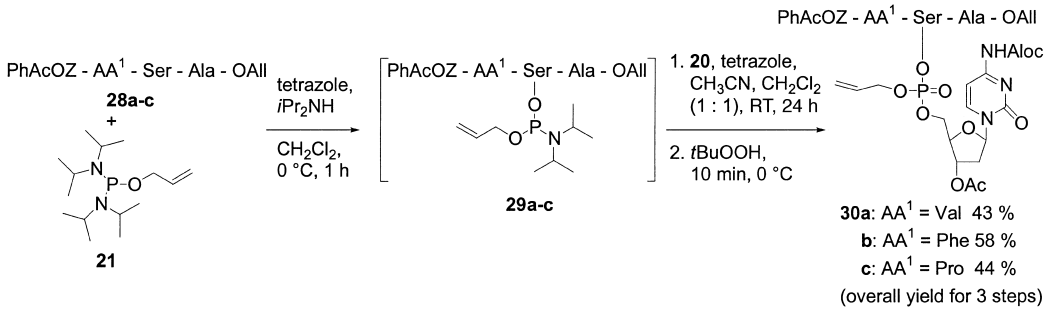
The central serine of tripeptides **28a–c** were then coupled to selectively masked deoxycytidine **20** through a phosphotriester bond. Upon treatment of **28a–c** with phosphordiamidite **21** in the presence of tetrazole and diisopropylamine, hydrolysis-sensitive intermediates **29a–c** were formed. These were converted without isolation into the desired peptide conjugates **30a–c** by the method described above in Scheme 4. The product was purified by gel permeation chromatography after filtration through silica gel to yield the nucleotripeptides **30a–c** in high yields in overall three steps (Scheme 6).

Enzymatic cleavage of the PhAcOZ-protecting group: Completely masked nucleotripeptides **30a–c** were then subjected to selective enzymatic deprotection with penicillin G acylase under the conditions described above. The enzyme smoothly cleaved the phenylacetic acid ester and the selectively unmasked nucleotripeptides **31a–c** were formed in high yield (Scheme 7). The immobilized enzyme was filtered and the



Scheme 7. Selective PGA-catalyzed removal of the N-terminal PhAcOZ urethane from nucleotripeptides **30a–c** and tetrapeptides **32a,b**.

solution was lyophilized. The phosphate salts and KI were separated from the desired compound by using a C18 Sep-Pak cartridge, as described above. Then the mixtures containing **31a–c** were separated by HPLC to yield the desired compounds in high yield. To assure that this method is widely applicable, the peptide chains of compounds **31a** and **31b** were elongated with a further PhAcOZ-protected amino acid in the presence of HATU and diisopropylethyl amine at 0 °C to yield nucleotetrapeptides **32a** and **32b** in 40 and 42%, respectively (Scheme 7). Once more, treatment of these peptide conjugates with PGA under the conditions described above resulted in a smooth and clean deprotection of the *N*-terminus to yield selectively deprotected nucleotetrapeptides



Scheme 6. Synthesis of model *N*-PhAcOZ-protected nucleotripeptides **30a–c**.

33a and **33b**. These nucleotetrapeptides were purified by the method described above.

The *N*-terminally deprotected nucleopeptides **31a**, **31b**, **33a** and **33b** can be converted to the fully deprotected nucleopeptides by Pd^0-catalyzed removal of the allyl groups. The removal of the allyl blocking groups from nucleopeptides under nonbasic conditions and without undesired decomposition reactions has been demonstrated by us[23a] and Hayakawa et al.[23b]

In all enzyme-catalyzed deprotections, undesired side reactions did not occur. Thus, the high selectivity of PGA for the phenylacetic acid group guarantees that the peptide bonds, the allyl ester, the allyl urethane, the acetate protecting group, and the phosphate remain fully intact. The conditions of the enzymatic transformation are so mild (pH 6.8) that the glycosidic bond is not affected and β-elimination of the phosphate (which occurs at pH > 7) is not observed at all. These findings prove that the PhAcOZ group is an efficient protecting function for the selective synthesis of sensitive and multifunctional nucleopeptides.

Conclusion

We have developed a new mild enzymatic method to synthesize nucleopeptides through selective *N*-terminal deprotection. We also demonstrated the applicability of this method by performing *N*-terminal chain elongations followed by deprotection. Since the enzymatic cleavage is very mild (pH 6.8, room temperature)and highly selective, no side reaction was encountered during the deprotection reaction. This method will open up new opportunities to synthesize nucleopeptides of biological interest.

Experimental Section

General: ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on Bruker 250, Am 400, and DRX 500 spectrometers. Mass spectra were measured on a HPLC/ESI-MS system with the HP (Agilent, 1100 series) HPLC. FAB-MS was measured on a Funnigan MAT MS 70 spectrometer. Optical rotation was measured on Perkin–Elmer 341 polarimeter. Analytical chromatography was performed on Merck silica gel 60 F254 plates. Flash chromatography was performed on Baker silica gel (40–64 mm). HPLC purifications were made by varian (Pro Star 215). C18-HD RP-analytical and semi-preparative and preparative columns were purchased from Macherey–Nagel. Melting point was measured in a Büchi 510 melting point apparatus. Solvents were dried according standard procedures. All reactions except the enzymatic transformations were carried out under argon. PGA immobilized on Eupergit C was from Fluka. Sep-Pak C18 cartridges were purchased from Waters, USA. Compound **21** was purchased from Aldrich and distilled before the reaction.

***N-tert*-Butyloxycarbonyl-L-aspartic diallyl ester (7)**: Boc-Asp-OH (1.88 g, 8 mmol) was dissolved in THF (9 mL) and water was added dropwise until the solution became turbid. It was neutralized by the addition of Cs_2CO_3 (2.64 g, 16.2 mmol) in water (3 mL) at room temperature. After a few minutes, the solution was evaporated to dryness and the salt obtained was suspended in water (15 mL) and treated with allyl bromide (1.75 mL). The mixture was stirred for 4 h at room temperature. Evaporation of the solvent and excess reagent gave the crude material, which was dissolved in ethyl acetate and washed with 5% $NaHCO_3$ solution and water. The organic layer was dried over $MgSO_4$, filtered, and evaporated. The compound was purified by silica gel column chromatography to yield the fully protected aspartic acid **7** (1.9 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.85-5.75$ (m, 2H), 5.53–5.51 (d, $J = 8.36$ Hz, 1H), 5.24–5.13 (m, 4H), 4.54–4.49 (m, 5H), 2.96–2.75 (m, 2H), 1.35 (s, 9H); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta =$ 170.59, 170.45, 155.26, 131.65, 131.49, 118.41, 79.84, 77.34, 66.05, 65.46, 49.94, 36.59, 28.16.

L-Aspartic diallyl ester, TFA salt (8): Trifluoroacetic acid (6 mL) was added to a solution of **7** (1.2 g, 3.8 mmol) in dichloromethane (50 mL), and the solution was stirred for 24 h at room temperature. Evaporation of the solvent and excess TFA gave the desired product **8** in quantitative yield. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 8.80-8.40$ (br s, 1H), 5.91–5.80 (m, 2H), 5.34–5.24 (m, 4H), 4.72–4.59 (m, 5H), 4.43–4.40 (t, $J = 4.84$, 9.84 Hz, 1H), 3.18–3.17 (d, $J = 4.44$ Hz, 2H); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 170.20$, 167.78, 130.98, 130.37, 120.06, 119.32, 67.68, 66.56, 49.59, 33.34.

***N-tert*-Butyloxycarbonyl-L-seryl-L-aspartic diallyl ester (10)**: EEDQ (4.0 g, 16 mmol) was added to a solution of Boc-Ser-OH (**9**) (1.6 g, 8 mmol) in dichloromethane (30 mL) at 0 °C. A solution of compound **8** (2.6 g, 8 mmol) in dichloromethane (10 mL) and then triethylamine (1.2 mL, 8 mmol) were added at 0 °C. The reaction mixture was stirred at RT overnight, washed three times with 0.1 M HCl, once with saturated $NaHCO_3$ solution and once with brine. The organic layer was dried over $MgSO_4$ and evaporated in vacuo. The crude material was purified by silica gel flash chromatography to yield dipeptide **10** (2.75 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.62$ (d, $J = 7.8$ Hz, 1H), 5.93–5.83 (m, 2H), 5.74 (d, $J = 7.2$ Hz, 1H), 5.34–5.23 (m, 4H), 4.94–4.90 (m, 1H), 4.65–4.57 (m, 4H), 4.25 (br s, 1H), 3.99 (br s, 1H), 3.69 (br s, 2H), 3.07–2.89 (m, 2H), 2.64 (s, 1H), 1.44 (s, 9H); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 171.27$, 170.45, 170.27, 131.61, 131.35, 118.99, 118.82, 80.26, 66.55, 65.83, 63.00, 55.50, 48.84, 36.09, 28.28; MS (FAB): *m*/*z* calcd: 400.42; found: 401.2 (3-NBA matrix).

L-Seryl-L-aspartic diallyl ester, TFA salt (11): The deprotection of the Boc-group was carried out as described above for **8** to afford **11**. ^{1}H NMR (400 MHz, CD_3OD): $\delta = 5.98-5.88$ (m, 2H), 5.36–5.29 (m, 2H), 5.25–5.21 (m, 2H), 4.64–4.59 (m, 5H), 3.99–3.95 (m, 2H), 3.83–3.78 (m, 1H), 2.96 (d, $J = 5.8$ Hz, 2H); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 171.38$, 171.22, 168.21, 133.23, 132.95, 122.90, 118.99, 118.75, 67.35, 66.69, 61.65, 56.07, 50.41, 36.65; MS (FAB): *m*/*z* calcd: 300.31; found: 301.2 (3-NBA matrix).

***N*-Phenyacetoxybenzyloxycarbonyl-L-aspartic β-allyl ester (14)**: Triethylamine (7.5 mmol) and trimethylsilyl chloride (TMSCl) (1.63 g, 15 mol) were added in one portion to a vigorously stirred suspension of **13** (1.57 g, 7.5 mmol) in CH_2Cl_2 (25 mL). The mixture was heated at reflux for 1 h, cooled to 0 °C, and diisopropylethylamine (2.2 mL, 13 mmol) and PhAcOZ-Cl (1.5 g, 5 mmol) were added. The reaction mixture was stirred at 0 °C for 30 min and at RT for 2 h. The solvent was removed in vacuo, and the residue was taken up in Et_2O (25 mL) and 0.5 M $NaHCO_3$ solution (50 mL). The layers were separated, and the aqueous layer was extracted with Et_2O (10 mL). The combined organic layers were washed with H_2O (10 mL). The pH of the combined aqueous layers was adjusted to pH 2 with HCl (0.5 M), and the aqueous solution was extracted with ethyl acetate. The combined organic layers were then dried with $MgSO_4$ and concentrated to give the desired product **14** (1.55 g, 70%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 10.6-10.4$ (br s, 1H), 7.4–7.1 (m, 7H), 7.1–7.0 (m, 2H), 5.9–5.7 (m, 2H), 5.3–5.2 (m, 2H), 5.1–5.0 (m, 2H), 4.7–4.5 (m, 4H), 3.9 (s, 2H), 3.1–2.8 (m, 2H); MS (FAB): *m*/*z* calcd: 441.43; found: 442.2 (3-NBA matrix).

***N*-Phenyacetoxybenzyloxycarbonyl-β-allyl-L-aspartyl-L-seryl-L-aspartic diallyl ester (15)**: A solution of **11** (0.22 g, 0.52 mmol) in CH_2Cl_2 (5 mL) was added to a solution of PhAcOZ-Asp(OAll)-OH (**14**) (0.24 g, 0.52 mmol), HOBT (0.08 g, 0.5 mmol) and EDC (0.2 g, 1 mmol) in CH_2Cl_2 (5 mL) at 0 °C. After addition of diisopropylethylamine (0.12 mL, 0.7 mmol), the mixture was stirred for 18 h at RT and washed with 0.1 M HCl (2 × 20 mL) and saturated NaCl (10 mL). Drying over $MgSO_4$ and evaporation of the solvent gave the desired product **15** (0.19 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J = 8$ Hz, 1H), 7.56 (d, $J = 7.1$ Hz, 1H), 7.43–7.18 (m, 7H), 7.02 (d, $J = 8.5$ Hz, 2H), 6.18 (d, $J = 8.7$ Hz, 1H), 5.96–5.80 (m, 3H), 5.41–5.18 (m, 7H), 5.18–5.00 (m, 2H), 4.95–4.85 (m, 1H), 4.67–4.43 (m, 7H), 4.03–3.90 (m, 1H), 3.84 (s, 2H), 3.78–3.57 (m, 2H), 3.01–2.64 (m, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 171.16$, 170.88, 170.41, 170.20, 170, 155.98, 150.54, 133.73, 133.32, 131.65, 131.37, 129.41, 129.30, 128.92, 128.73, 128.49, 127.38, 126.54, 121.59, 118.89, 118.72, 118.67, 77.45, 77.13, 76.81, 66.55, 66.46, 65.79, 62.66, 54.73, 51.26, 48.86, 41.33, 36.55, 35.99; MS (FAB): *m*/*z* calcd: 723.72; found: 724.3 (3-NBA matrix).

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5′-*O*-(*tert*-Butyldimetylsilyl)-3′-*O*-acetyl-4-*N*-allyloxycarbonyl-2′-deoxycytidine (19): A solution of **18** (0.1 g, 0.23 mmol) and acetic anhydride (0.25 mL) in pyridine (5 mL) was stirred for 3 h at room temperature. Evaporation of excess acetic anhydride and pyridine gave a foam, and purification by chromatography on silica gel (chloroform/methanol 10:0.5) afforded **19** (0.106 g, 99 %). $R_f = 0.73$ (CH_2Cl_2/MeOH 20:1); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.20$ (d, $J = 7.55$ Hz, 1H), 7.13 (br s, 1H), 6.28 (dt, $J = 5.7$ Hz, 1H), 5.92–5.84 (m, 1H), 5.31 (dd, $J = 17.1$, 1.3 Hz, 1H), 5.23–5.18 (m, 2H), 4.63 (d, $J = 5.6$ Hz, 2H), 4.14 (d, $J = 1.85$ Hz, 1H), 3.85 (dq, $J = 11.4$, 16.1 Hz, 2H), 2.65 (dd, $J = 13$ Hz, 1H), 2.08–2.01 (m, 1H), 2.03 (s, 3H), 0.83 (s, 9H), 0.04 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 176.96$, 170.50, 162.53, 144.14, 131.47, 118.89, 87.10, 86.04, 75.19, 66.68, 63.33, 39.48, 25.82, 21.18, 20.95, 18.21, −5.52, −5.63.

3′-*O*-Acetyl-4-*N*-alloxycarbonyl-2′-deoxycytidine (20): 1M TBAF (0.4 mL) in THF was added to a solution of **19** (0.1 g, 0.21 mmol) in THF (6 mL) at room temperature, and the mixture was stirred for 3 h at room temperature. After quenching with methanol, the solvent was evaporated in vacuo. The residual foam was purified by chromatography on silica gel (chloroform/methanol/triethylamine 100:5:0.5) to give **20** (0.07 mg, 98 %) as a white solid. $R_f = 0.1$ (CH_2Cl_2/MeOH 20:1); $[\alpha]_D^{22} = +43.2$ ($c = 1.0$ in MeOH); m.p. 164 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.34$ (d, $J =$ 7.5 Hz, 1H), 7.27 (s, 1H), 6.27 (t, $J = 6.6$ Hz, 1H), 5.97–5.89 (m, 1H), 5.39–5.35 (m, 2H), 5.30 (d, $J = 10.3$ Hz, 1H), 4.68 (d, $J = 5.4$ Hz, 2H), 4.19 (s, 1H), 3.95 (q, $J = 12.1$, 16.3 Hz, 2H), 3.70–3.40 (br s, 1H), 2.67 (dd, $J =$ 4.12 Hz, 1H), 2.38–2.31 (m, 1H), 2.1 (s, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 170.84$, 162.37, 145.03, 131.34, 119.18, 95.50, 87.83, 86.08, 77.33, 77.01, 76.70, 74.87, 66.83, 62.20, 38.71, 21.00; MS (FAB): *m*/*z* calcd: 353.33; found: 354.2 (3-NBA matrix).

Synthesis of nucleotripeptide 23

Synthesis of the phosphoramidite (22): A solution of **15** (0.72 g, 1 mmol) in CH_2Cl_2 (1 mL) was added to a solution of allyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (**21**) (1.3 mmol, 0.415 mL), diisopropylamine (0.5 mmol, 0.071 mL), and tetrazole (35 mg, 0.5 mmol) in CH_2Cl_2 (2 mL). After 1 h, the reaction mixture was taken up in a saturated aqueous solution of $NaHCO_3$ (5 mL). The mixture was extracted four times with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. The crude products were used immediately for subsequent steps without further purification.

***N*-Phenyacetoxybenzyloxycarbonyl-*β*-allyl-L-aspartyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-aspartic diallyl ester (23)**: A solution of tetrazole (0.14 g, 2 mmol) in acetonitrile (2 mL) at room temperature was added dropwise to a solution of **20** (1 mmol) and the crude **22** in CH_2Cl_2 (2 mL). After completion of the reaction (24 h), *t*BuOOH (80 %, 2 mL) was added at 0 °C. After 10 min, the reaction mixture was taken up in water (100 mL), and the solution was extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$ and concentrated, and the residual oil was purified by chromatography on silica gel (chloroform/ethanol 20:1) and gel filtration chromatography to give **23** (0.475 g, 40 %). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 8.09$, 8.04 (2d, $J = 7.2$ Hz, 1H), 7.99, 7.96 (2d, $J = 7.6$ Hz, 1H), 7.84, 7.79 (2d, $J = 8$ Hz, 1H), 7.40–7.20 (m, 7H), 7.03, 7.025 (2d, $J = 8$ Hz, 2H), 6.34–6.31 (m, 1H), 6.21–6.16 (m, 1H), 5.96–5.82 (m, 5H), 5.37–5.19 (m, 12H), 5.11–5.02 (m, 2H), 4.91–4.85 (m, 2H), 4.78–4.74 (m, 1H), 4.65–4.45 (m, 12H), 4.35–4.28 (m, 3H), 4.22 (d, $J = 2.3$ Hz, 1H), 3.85 (s, 2H), 3.04–2.85 (m, 4H), 2.60–2.56 (m, 1H), 2.24–2.16 (m, 1H), 2.06 (s, 3H); ^{13}C NMR (125.77 MHz, $CDCl_3$): $\delta =$ 171.20, 171.13, 170.91, 170.51, 170.21, 170.13, 169.93, 169.86, 168.21, 168.11, 162.71, 156.00, 150.56, 144.41, 144.29, 133.83, 133.36, 132.11, 132.06, 131.77, 131.74, 131.53, 131.48, 129.30, 129.26, 128.93, 128.74, 127.39, 121.59, 119.03, 118.68, 118.57, 95.61, 87.87, 87.51, 83.44, 77.34, 77.09, 76.83, 74.21, 74.09, 68.95, 68.90, 67.17, 67.05, 66.90, 66.70, 66.47, 66.29, 65.72, 65.66, 53.34, 51.36, 49.08, 41.36, 38.14, 38.06, 36.55, 35.98, 20.88; ^{31}P NMR (202.48 MHz, $CDCl_3$): $\delta = -0.44$; MS (FAB): *m*/*z* calcd: 1179.08; found: 1179.2 (3-NBA matrix)

Synthesis of the nucleotripeptides (28a–c)

***N*-Phenyacetoxybenzyloxycarbonyl-L-valinyl-L-seryl-L-alanine allyl ester (28a)**: A solution of **26a** (0.5 mmol, 0.19 g) and HATU (0.6 mmol, 0.23 g) in dry DMF (5 mL) was stirred for 5 min at 0 °C. Then, a solution of the dipeptide trifluoroacetic acid ester **27** (0.7 mmol, 0.235 g) in dry DMF (5 mL), and diisopropylamine (1.2 mmol, 0.2 mL) were added. After stirring for 1 h, the reaction mixture was diluted with CH_2Cl_2 and washed once with saturated $NaHCO_3$ solution and 1M HCl. The organic layer was dried over $MgSO_4$ and concentrated, and the residue was purified by chromatography on silica gel to yield **28a** (0.26 g, 88 %). $R_f = 0.24$ (ethylacetate/cyclohexane 3:1); $[\alpha]_D^{22} = -17.6$ ($c = 1.0$ in CH_2Cl_2); m.p. 150 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.38-7.20$ (m, 9H), 7.02 (d, $J =$ 8.3 Hz, 2H), 5.93–5.83 (m, 1H), 5.75 (d, $J = 8$ Hz, 1H), 5.35–5.20 (m, 2H), 5.08–5.00 (m, 2H), 4.66–4.51 (m, 4H), 4.17–4.10 (m, 1H), 3.97–3.95 (br d, $J = 10.8$ Hz, 1H), 3.85 (s, 2H), 3.67–3.59 (m, 1H), 2.15–2.02 (m, 1H), 1.40 (d, $J = 7$ Hz, 3H), 0.95 (d, $J = 6.8$ Hz, 3H), 0.90 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 172.39$, 172.01, 170.23, 169.99, 150.46, 133.88, 133.28, 131.40, 129.27, 128.72, 127.36, 121.54, 118.83, 66.33, 66.09, 62.65, 60.26, 53.98, 48.34, 41.34, 37.29, 31.22, 19.15, 17.78, 17.71, −0.03; MS (ESI+): *m*/*z* (%) calcd: 583.63; found: 584.10 (100). Compounds **28b** and **28c** were prepared by the same procedure.

***N*-Phenyacetoxybenzyloxycarbonyl-L-phenlyalanyl-L-seryl-L-alanine allyl ester (28b)**: Yield = 75 %; solid; m.p. 115 °C; $R_f = 0.15$ (ethylacetate/cyclohexane 3:1); $[\alpha]_D^{22} = -12.6$ ($c = 1$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.40-7.12$ (m, 13H), 7.02–6.97 (m, 2H), 5.92–5.78 (m, 2H), 5.34–5.20 (m, 2H), 5.04–4.92 (m, 2H), 4.64–4.48 (m, 6H), 3.92–3.84 (m, 1H), 3.84 (s, 2H), 3.76–3.68 (m, 1H), 3.64–3.56 (m, 1H), 3.14–3.06 (m, 1H), 3.02–2.94 (m, 1H), 1.44–1.36 (m, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 172.86$, 172.65, 172.09, 170.23, 170.20, 156.29, 150.72, 136.41, 134.05, 133.56, 131.70, 129.514, 129.47, 129.43, 128.96, 128.85, 127.60, 127.26, 121.76, 119.02, 66.54, 66.31, 63.00, 54.61, 48.64, 41.59, 38.74, 17.89; MS (ESI+): *m*/*z* (%) calcd: 631.68; found: 632.10 (100).

***N*-Phenyacetoxybenzyloxycarbonyl-L-prolyl-L-seryl-L-alanine allyl ester (28c)**: Yield = 85 %; white wax; $R_f = 0.04$ (ethylacetate/cyclohexane 3:1); $[\alpha]_D^{22} = -50.2$ ($c = 0.5$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.40-$ 7.20 (m, 9H), 7.03 (d, $J = 6.4$ Hz, 2H), 5.92–5.84 (m, 1H), 5.31 (d, $J =$ 13.8 Hz, 1H), 5.23 (d, $J = 8.4$ Hz, 1H), 5.20–4.95 (m, 2H), 4.64–4.46 (m, 4H), 4.34–4.28 (m, 1H), 4.25–4.20 (br s, 0.5H), 3.98–3.97 (m, 0.5H), 3.84 (s, 2H), 3.76–3.64 (m, 1H), 3.60–3.50(m, 1H), 3.48–3.42 (m, 1H), 3.17–3.02 (br s, 1H), 2.20–2.02 (m, 2H), 1.99–1.82 (m, 2H), 1.41–1.30 (m, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 173.35$, 172.88, 172.80, 175.58, 170.49, 156.06, 155.09, 150.85, 134.62, 134.42, 133.70, 132.00, 129.69, 129.60, 129.13, 127.78, 121.97, 119.11, 118.98, 67.12, 66.90, 66.33, 63.04, 61.37, 60.93, 55.08, 45.52, 48.80, 47.90, 47.54, 41.73, 31.68, 30.15, 24.91, 24.04, 17.91; MS (ESI+): *m*/*z* (%) calcd: 381.61; found: 382.10 (100).

Preparation of comopunds 30a–c: Compounds **30a–c** were prepared by the procedure described above for compound **23**.

***N*-Phenylacetoxybenzyloxycarbonyl-L-valinyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine-allyl ester (30a)**: Yield = 43 %; white solid; m.p. 68 °C; $R_f = 0.17$ ($CHCl_3$/EtOH 20:1); $[\alpha]_D^{22} = +3.6$ ($c = 0.5$ in CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 8.10–7.94 (m, 2H), 7.73 (d, $J = 7.0$ Hz, 0.5H), 7.67 (d, $J = 7.0$ Hz, 0.5H), 7.38–7.24 (m, 7H), 7.04 (d, $J = 8.5$ Hz, 2H), 6.24–6.20 (m, 1H), 5.96–5.80 (m, 3H), 5.41–5.20 (m, 8H), 5.10–4.97 (m, 5H), 4.68–4.52 (m, 9H), 4.40–4.26 (m, 2H), 4.24–4.19 (m, 1H), 3.86 (s, 2H), 2.61–2.51 (m, 1H), 2.25–2.10 (m, 3H), 2.07 (s, 3H), 1.42–1.40 (d, $J = 7.3$ Hz, 3H), 0.97, 0.96 (2d, $J =$ 6.5 Hz, 3H), 0.91, 0.905 (2d, $J = 6.8$ Hz, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 172.56$, 172.49, 172.38, 170.96, 170.94, 170.34, 168.74, 168.54, 163.36, 156.86, 155.84, 152.86, 150.91, 144.18, 134.36, 133.73, 132.35, 132.04, 131.92, 129.60, 129.12, 127.77, 121.98, 119.52, 119.43, 119.25, 118.98, 118.94, 96.23, 87.34, 83.82, 83.74, 74.45, 74.36, 69.30, 69.24, 67.59, 67.22, 67.00, 66.94, 66.66, 66.25, 60.29, 53.21, 48.80, 41.74, 38.68, 38.59, 31.76, 31.70, 21.31, 21.29, 19.65, 18.23, 18.19, 17.85, 17.79; ^{31}P NMR (202.46 MHz, $CDCl_3$): $\delta = 0.0381$, 0.0016; MS (ESI+): *m*/*z* (%) calcd: 1038.99; found: 1039.10 (100).

***N*-Phenylacetoxybenzyloxycarbonyl-L-phenylalanyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine allyl ester (30b)**: Yield = 58 %; solid; m.p. 55 °C; $R_f = 0.13$ ($CHCl_3$/EtOH 20:1); $[\alpha]_D^{22} = +8.0$ ($c = 0.35$ in CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.60-8.40$ (br s, 1H), 7.81–7.66 (br s, 1H), 7.43–7.13 (m, 13H), 7.00 (d, $J = 7.5$ Hz, 2H), 6.26–6.12 (m, 1H), 5.99–5.72 (m, 4H), 5.45–5.17 (m, 7H), 5.06–4.20 (m, 16H), 3.85 (s, 2H), 3.70–3.55 (m, 1H), 3.50–3.35 (m, 1H), 3.25–3.15 (m, 1H), 3.02–2.90 (m, 1H), 2.78–2.64 (m, 1H), 2.50–2.30 (m, 1H), 2.06 (s, 3H), 1.44 (d, $J = 7.3$ Hz, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 172.37$, 172.23, 170.97, 170.33, 168.45, 168.29, 163.22, 156.36, 152.77, 150.87, 144.30, 136.80, 134.26, 133.73, 132.42, 132.35, 132.06, 131.85, 129.69, 129.51, 129.13, 128.91, 127.78, 127.28, 121.94, 119.48, 119.31, 118.98, 118.93, 96.14, 96.1, 87.82, 87.55, 83.79, 74.48, 69.33, 69.28, 67.60, 67.56, 67.35,

67.15, 67.05, 67.00, 66.61, 66.25, 56.41, 52.32, 48.82, 41.75, 38.95, 38.73, 21.31, 21.28, 18.16, 18.11; MS (ESI+): *m/z* (%) calcd: 1087.04; found: 1087.1 (100).

***N*-Phenylacetoxybenzyloxycarbonyl-L-prolyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine allyl ester (30c)**: Yield = 44%; white solid; m.p. 60°C; R_f = 0.15 ($CHCl_3$/EtOH 20:1); $[\alpha]_D^{22} = -11.8$ (c = 0.5 in CH_3OH); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.07–7.88 (m, 2H), 7.76–7.63 (m, 1H), 7.40–7.20 (m, 7H), 7.04 (d, J = 8 Hz, 2H), 6.30–6.24 (m, 1H), 5.97–5.83 (m, 3H), 5.40–5.00 (m, 9H), 4.93–4.85 (br s, 1H), 4.70–4.18 (m, 5H), 3.86 (s, 2H), 3.68–3.58 (m, 1H), 3.52–3.42 (m, 1H), 2.70–2.60 (m, 1H), 2.20–1.84 (m, 9H), 1.42 (d, J = 6.8 Hz, 3H), 1.46–1.32 (m, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 172.48, 172.38, 170.94, 170.34, 168.73, 168.53, 163.35, 156.85, 155.84, 152.85, 150.90, 144.18, 134.36, 133.73, 132.35, 132.04, 131.91, 129.69, 129.59, 129.27, 129.12, 127.77, 121.98, 119.52, 119.43, 119.25, 118.98, 118.94, 96.23, 87.73, 87.34, 83.82, 83.74, 74.45, 74.36, 69.30, 69.24, 67.59, 67.22, 67.00, 66.94, 66.66, 66.25, 60.28, 53.20, 48.80, 41.74, 38.67, 38.59, 31.76, 31.70, 26.16, 21.30, 19.65, 18.23, 18.19, 17.85, 17.79; MS (ESI+): *m/z* (%) calcd: 1036.97; found: 1037.10 (100).

***N*-Terminal deprotection of the nucleopeptides 30 and 32 by PGA**: A solution of the respective PhAcOZ-protected nucleopeptide (20–30 mg, 0.02 mmol) in a mixture of MeOH (12 mL), Na_2HPO_4 buffer (0.05 M, 40 mL) and KI (0.1 M, 10 mL) was treated with PGA (800 mg) at pH 6.8 for 24 h at room temperature. The immobilized enzyme was filtered, and the solution was lyophilized. The residue was dissolved in the minimum amount of water required (ca. 1 mL) and filtered through a C18 cartridge. The cartridge was washed with water (2 mL), and the absorbed organic materials were eluted with MeOH (2 mL). After evaporation of the solvent, the crude material was purified by HPLC (C18-HD RP).

L-Valinyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine allyl ester (31a): Yield = 60%; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.15 (2d, J = 7.8 Hz, 1H), 7.30, 7.29 (2d, J = 7.8 Hz, 1H), 6.22–6.15 (m, 1H), 6.05–5.85 (m, 3H), 5.43–5.19 (m, 7H), 4.78–4.74 (m, 1H), 4.69 (d, J = 5.8 Hz, 2H), 4.64–4.54 (m, 4H), 4.50–4.23 (m, 6H), 3.82, 3.79 (2d, J = 5.8 Hz, 1H), 2.72–2.64 (m, 1H), 2.41–2.32 (m, 1H), 2.27–2.18 (m, 1H), 2.12, 2.11 (2s, 3H), 1.42, 1.415 (2d, J = 7.3 Hz, 3H), 1.07 (d, J = 6.8 Hz, 3H), 1.04 (d, J = 6.8 Hz, 3H); ^{31}P NMR (202.46 MHz): δ = −0.41, −0.51; MS (ESI+): *m/z* (%) calcd: 770.72; found: 771.10 (100).

L-Phenylalanyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine allyl ester (31b): Yield = 69%; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.20–8.10 (m, 1H), 7.38–7.20 (m, 6H), 6.22–6.13 (m, 1H), 6.04–5.85 (m, 3H), 5.43–5.16 (m, H), 4.74–4.20 (m, 15H), 3.09–3.00 (m, 1H), 2.71–2.61 (m, 1H), 2.41–2.30 (m, 1H), 2.11, 2.10 (2s, 3H), 1.44, 1.42 (2d, J = 7.2 Hz, 3H). MS (ESI+): *m/z* (%) calcd: 818.76; found: 819.10 (100).

L-Prolyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine allyl ester (31c): Yield = 65%; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.17, 8.15 (2d, J = 7.5 Hz, 1H), 7.26, 7.24 (2d, J = 7.5, 1H), 6.18–6.14 (m, 1H), 6.04–5.87 (m, 3H), 5.43–5.19 (m, 7H), 4.77–4.73 (m, 1H), 4.70–4.67 (m, 2H), 4.63–4.56 (m, 4H), 4.49–4.25 (m, 7H), 3.48–3.40 (m, 1H), 3.40–3.33 (m, 1H), 2.71–2.66 (m, 1H), 2.50–2.34 (m, 2H), 2.11, 2.12 (2s, 3H), 2.10–2.02 (m, 3H), 1.43, 1.41 (2d, J = 2.8 Hz, 3H); MS (ESI+): *m/z* (%) calcd: 768.71; found: 769.3 (100).

Coupling of 31a,b with PhAcOZ-protected amino acid: A solution of the PhAcOZ-protected amino acid (0.0078 mmol) and HATU (6 mg, 0.0156 mmol) in dry DMF (1 mL) was stirred for 5 min at 0°C. Then, a solution of the **31a**,**b** (0.0052 mmol) in dry DMF (1 mL), and diisopropylamine (2 drops) were added. After stirring for 1 h, the reaction mixture was concentrated under reduced pressure and purified by HPLC.

***N*-Phenylacetoxybenzyloxycarbonyl-L-prolyl-L-valinyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine-allyl ester (32a)**: Yield = 85%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02–7.96 (m, 1H), 7.70–7.60 (m, 1H), 7.43–7.22 (m, 8H), 7.07–7.02 (m, 2H), 6.22–6.15 (m, 1H), 5.98–5.83 (m, 3H), 5.40–5.00 (m, 11H), 4.80–4.20 (m, 14H), 3.87 (s, 2H), 3.88–3.84 (m, 1H), 3.60–3.40 (m, 4H), 2.70–2.60 (m, 1H), 2.36–2.20 (m, 2H), 2.09 (s, 3H), 2.04–1.88 (m, 2H), 1.42 (d, J = 6.4 Hz, 3H), 0.94–0.80 (m, 6H); MS (ESI+): *m/z* (%) calcd: 1136.10; found: 1136.20, 1158 (100) $[M+Na]^+$.

***N*-Phenylacetoxybenzyloxycarbonyl-L-alanyl-L-phenylalanyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine-allyl ester (32b)**: Yield = 91%; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.19 (d, J = 7.5 Hz, 0.5H), 8.14 (d, J = 7.8 Hz, 0.5H), 7.60 (d, J = 7.3 Hz, 1H), 7.39–7.13 (m, 13H), 7.06–7.02 (m, 3H), 6.20–6.12 (m, 1H), 5.98–5.82 (m, 3H), 5.46–5.21 (m, 8H), 5.12–4.94 (m, 3H), 4.71–4.67 (m, 4H), 4.63–4.59 (m, 2H), 4.56–4.46 (m, 3H), 4.45–4.37 (m, 1H), 4.35–4.24 (m, 4H), 4.17–4.10 (m, 2H), 3.86 (s, 2H), 3.28–3.18 (m, 1H), 3.07–2.98 (m, 1H), 2.71–2.63 (m, 1H), 2.35–2.23 (m, 1H), 2.10, 2.09 (2s, 3H), 1.42, 1.41 (2d, J = 7.3 Hz, 3H), 1.27–1.24 (m, 3H); MS (ESI+): *m/z* (%) calcd: 1158.11; found: 1158.10 (100).

L-Alanyl-L-phenylalanyl-L-seryl-*O*-(3′-*O*-acetyl-6-*N*-allyloxycarbonyl-2′-deoxycytidyl-allyl-phosphato)-L-alanine-allyl ester (33b): Yield = 40%; ^{1}H NMR (400 MHz, CD_3OD): δ = 8.19, 8.18 (2d, J = 7.5 Hz, 1H), 7.33, 7.32 (2d, J = 7.5 Hz, 1H), 7.27–7.20 (m, 5H), 6.22–6.18 (m, 1H), 6.03–5.87 (m, 3H), 5.43–5.20 (m, 7H), 4.70–4.56 (m, 8H), 4.46–4.23 (m, 6H), 3.87–3.83 (m, 1H), 3.21, 3.17 (2d, J = 5.5 Hz, 1H), 3.00–2.92 (m, 1H), 2.71–2.62 (m, 1H), 2.37–2.25 (m, 1H), 2.11 (s, 3H), 1.48 (d, J = 7.0 Hz, 3H), 1.41 (d, J = 7.3 Hz, 3H); (ESI+): *m/z* (%) calcd: 889.84; found: 890.2 (100).

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