Synthesis of a Pyridoxine–Peptide Based Delivery System for Nucleotides

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As a first step in the development of a nucleotide delivery system making use of oligopeptide permease, we have synthesized pyridoxine–peptide–nucleotide conjugates. The nucleotides are bonded on serine residues. The peptides terminate with a pyroglutamate residue. In the first example, the pyridoxine moiety is connected at the end of the peptides, while, in the second example, the pyridoxine moiety is bonded at an aspartic acid residue in a middle position of the peptide. Nucleotides are introduced as phosphoramidites. The synthetic strategy involves a series of protection, deprotection, and coupling reactions, and integrates peptide, nucleotide, and pyridoxine chemistry. The final deprotection step was carried out in basic conditions using 10% K_2CO_3 in MeOH.

Introduction. – Nucleoside mono-, di-, and triphosphates penetrate cell membranes poorly, limiting their use as substrates or inhibitors for enzymes in the cell. Nucleosides themselves, however, are easily taken up by cells. Within the cell, they can be converted to mono-, di-, and triphosphates by the action of nucleoside and nucleotide kinases. However, most modified nucleosides are poorly (if at all) phosphorylated in the cells. Several phosphorylated modified nucleosides have interesting properties *in vitro*, but are useless *in vivo*. The approaches that can be used to generate modified nucleotides in the cell are *a*) the use of prodrugs of nucleotides in which the phosphate protecting groups are removed after cell penetration; *b*) mutating intracellularly expressed kinases in order to broaden their substrate specificity; *c*) bind the nucleotide to a transporter molecule which can deliver its message into the cell. The first approach might work well for the delivery of nucleoside monophosphates but becomes very complicated for the delivery of triphosphates. The second approach implies the engineering of the appropriate kinases. The substrate specificity of kinases varies according to their origin. It is well-known, for example, that *E. coli* kinases have a narrow substrate specificity, while kinases from HSV-1 and from *D. melanogaster* have a much broader substrate specificity [1]. This substrate specificity can be manipulated by the induction of mutations in the enzymes. However, it remains a very complicated procedure to select the most appropriate kinase for every interesting modified nucleoside, and to transfect the host organism with the selected plasmid encoding for this kinase. The procedure would solve the problem for one modified nucleoside, but does not guarantee that the selected kinase is also the most efficient enzyme for another substrate. Moreover, for the intracellular synthesis of triphosphates, several

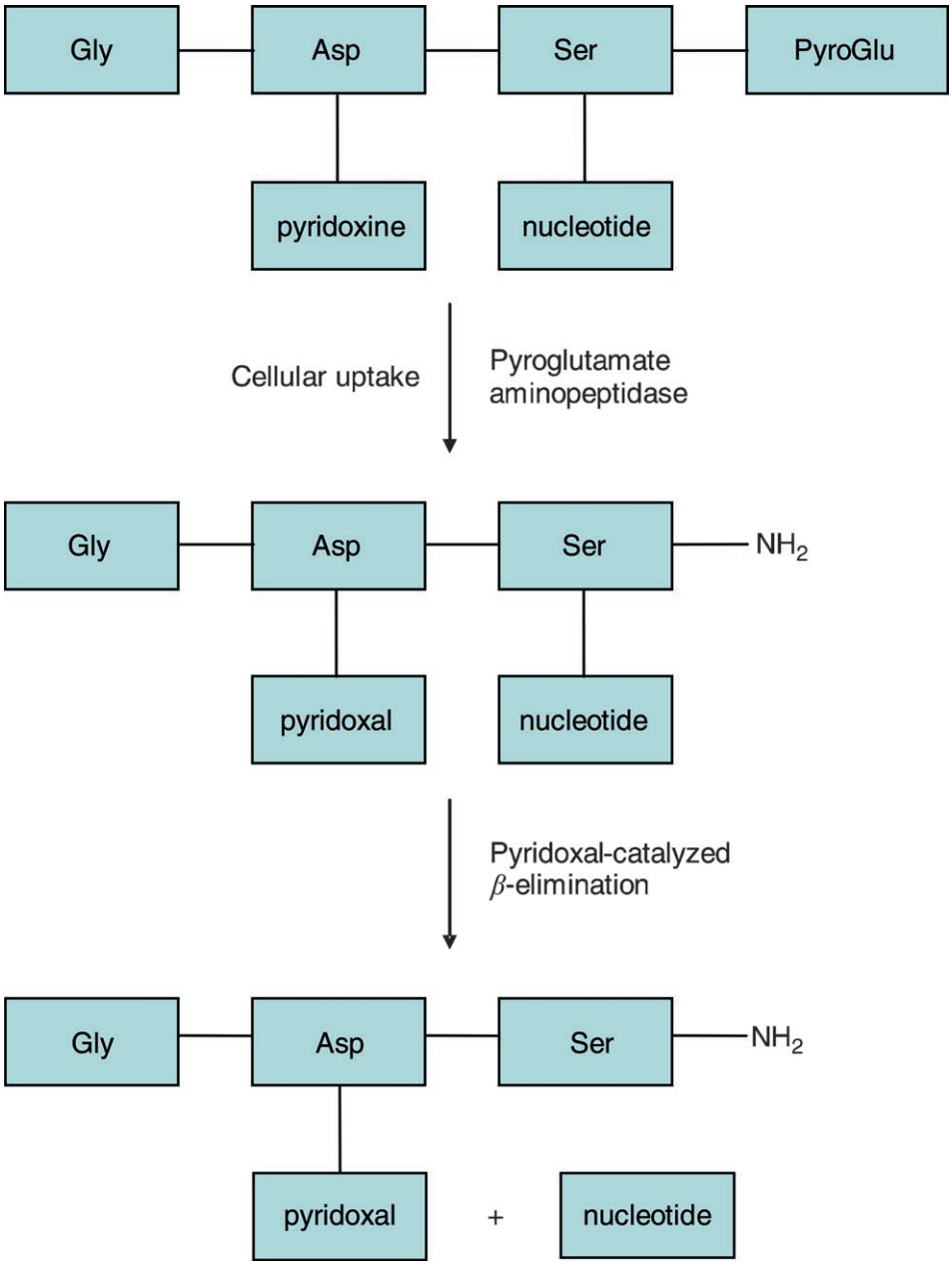
enzymes need to be reengineered. In case of the *in vivo* expansion of the genetic alphabet, for example, one would like to have an approach that works equally well for several modified bases and for three phosphorylation steps.

The third approach involves investigations on the development of an uptake system for nucleoside mono-, di-, and triphosphates so that their intracellular appearance becomes independent of the presence of the appropriate kinase in the cell. Our interest in this approach originates from research projects with respect to molecular evolution in *E. coli* using sugar-modified nucleotides and nucleic acids. The availability of such a delivery system might also be useful to study the toxicity of triphosphates of modified nucleosides in this organism.

The Molecular-Design Concept. Every organism needs to take up extracellular nutritients in order to become the necessary supply of materials and energy for cellular functioning. The transfer of organic and inorganic molecules from the outside of the cell into the cytoplasm are carried out by permeases. The specificity of the transport system is defined by the presence of periplasmic substrate-binding proteins, and they play a key role in the selection of compounds that can be taken up by cells. These binding-protein-dependent permeases are part of a much larger group of transport systems known as the ATP-binding cassette transporters [2]. The periplasmic binding proteins of *Gram*-negative species are proteins situated in the periplasmic space and are part of the oligopeptide permease system [3]. In *Gram*-positive bacteria, the proteins are anchored to the cell membrane by a lipophilic tail. The most versatile of these receptors is the one associated with oligopeptide permeases [4]. This periplasmic oligopeptide-binding protein (OppA) is the initial receptor for the uptake of peptides by the oligopeptide permease (Opp) in *Gram*-negative bacteria [5]. The structure of the protein was determined by X-ray diffraction studies [6]. The protein has a variable peptide core and a unique surface structure that is recognized by the membrane component of the transport system. The ligand-binding site of OppA is buried within the protein, and it shows a broad substrate specificity, *i.e.*, it accepts di- to pentapeptides regardless of the identity of the side chain [6]. The side-chain pockets in OppA are indeed rather unusual, because these cavities are spacious and hydrated, and there are few direct contacts between ligand and protein. Therefore, the protein will accept chemical groups of an enormous diversity. Because of this diversity, we would like to investigate the possibility of using this system for the transportation of nucleotides covalently linked to peptides. The feasibility of this '*Trojan horse*' approach [7] for the delivery of ligands covalently bonded to peptides has been demonstrated by using synthetic bacterial compounds [8–10].

It was postulated that the critical requirement for cell-uptake by the OppA is the presence of a positively charged N-terminus interacting with the side chain of Asp(419) [5]. However, the presence of a free amino group might facilitate extracellular degradation of the peptide conjugate by peptidases and/or cause premature disintegration of the molecule by the chemical mechanism described hereafter. For both reasons, we propose to protect the amino terminus of the peptide with a pyroglutamyl residue (*Scheme 1*). This derivatization is a common post-translational modification of proteins (and peptides). The *N*-terminal pyroglutamyl residue can be removed enzymatically with pyroglutamate aminopeptidase (PGAP). This enzyme has been identified in several bacterial, animal, and human tissues [11], and exists in two forms (a

Scheme 1. *Example of a Pyridine–Peptide Delivery System as Potential Substrate for Oligopeptide Permease*



soluble cytoplasmic form and a membrane bound form). The action of pyroglutamate aminopeptidase on the proposed conjugate should liberate the free terminal amino group that may initiate the cellular uptake of the construct as well as the intracellular delivery of the nucleotide.

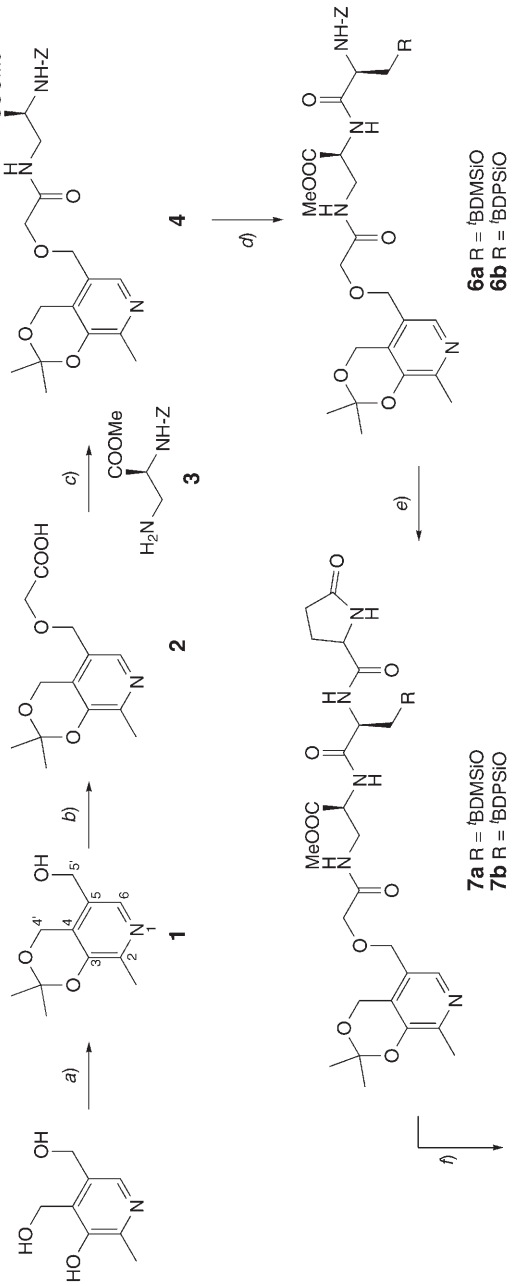
One of the most versatile catalysts in nature is pyridoxine (= 5-hydroxy-6-methylpyridine-3,4-dimethanol) or vitamin B_6. In its aldehyd form (pyridoxal), it may react with the primary amino group of amino acids and function as coenzyme in many metabolic reactions of amino acids (such as transamination, decarboxylation, elimination, racemization). The key mechanism of these transformations is the imine formation, followed by the delocalization of electrons from the amino acids into the pyridoxal ring. By this mechanism, every bond around the α-C-atom of the amino acid can be cleaved, and, in the absence of an enzyme, more than one degradation pathway is often observed. One of the key reactions catalyzed by vitamin B_6 is the formation of pyruvate from serine by an elimination–hydration process. In theory, this reaction can be used to eliminate (and delivers) molecules that are bound to the side-chain OH group of serine. The delivery vehicle has, therefore, a pyridoxine terminal (which needs to be oxidized to pyridoxal within the cell) and a serine residue loaded with the modified nucleotide that needs to be delivered.

In a first experiment, deoxythymidine 5′-monophosphate was selected as the delivery nucleotide. This nucleotide itself is not taken up by *E. coli*, but is needed for cell survival when using cells lacking the enzymes for its synthesis. In this way, cellular uptake and delivery process can easily be observed. The way all these elements need to be assembled in a molecular machinery in order to become efficient is the subject of this research project. As a first step, we investigated the general synthetic scheme to prepare this type of compounds. Several spacers connecting the pyridoxine group and the loaded serine need to be synthesized, and the elimination of the nucleotide under different conditions need to be investigated *in situ* (as well as the degradation process as the kinetics of the reactions). The pyridoxal group may also be replaced by other, related groups with similar characteristics.

Chemistry. – The synthetic scheme to obtain the delivery system for deoxythymidine 5′-monophosphate has been worked out (*cf. Schemes 2–4*). Pyridoxine is protected at its 3- and 4′-positions[1]) using an isopropylidene group in 75% yield [12] (*Scheme 2*). Reaction of 3,4′-*O*-isopropylidenepyridoxine (**1**) with $ClCH_2COOH$ in the presence of NaH under reflux yielded compound **2**. 2,3-Diaminopropanoic acid (Dap) was converted into its methyl ester **3** after protection of the 2-amino group with a (benzyloxy)carbonyl (Z) group (*Scheme 3*). Condensation of **2** with the protected methyl 2,3-diaminopropanoate **3** using 1,1′-carbonyldiimidazole gives compound **4** in 83% yield, from which the Z group was removed by hydrogenation. The second peptide bond is formed between compound **4** and appropriately protected serine. Therefore, Z-Ser-OH was first reacted with (*tert*-butyl)dimethylsilyl chloride (^{t}BDMSi–Cl) and 1*H*-imidazole in DMF to form **5a** (see *Scheme 4*). The condensation reaction was carried out using DCC and DMAP (*Scheme 2*). This reaction step was repeated with pyroglutamic acid as amino acid, *i.e.*, the Z group of **6** was first removed by

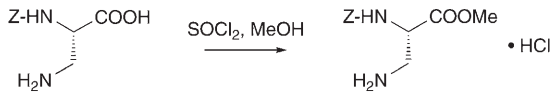
[1]) Pyridoxine numbering; see formula **1**.

Scheme 2



a) 2,2-Dimethoxypropane (DMP), H_2SO_4, CH_2Cl_2, reflux. *b*) NaH, $ClCH_2CO_2H$, THF, reflux. *c*) 1. 1,1′-Carbonyldiimidazole (CDI), CH_2Cl_2; 2. **3**, Et_3N. *d*) 1. H_2/Pd–C (10%), MeOH; 2. **5a**, **5b**, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), CH_2Cl_2. *e*) 1. H_2/Pd–C (10%), MeOH; 2. pyroglutamic acid, DCC, DMAP, MeCN. *f*) 1. Pyridinium *para*-toluenesulfonate (PPTS), EtOH, reflux; 2. Ac_2O, pyridine; 3. Bu_4NF (TBAF) (1M), THF, 0°.

Scheme 3



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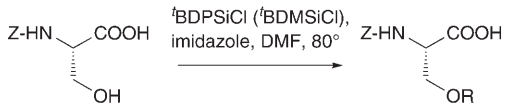
Scheme 4



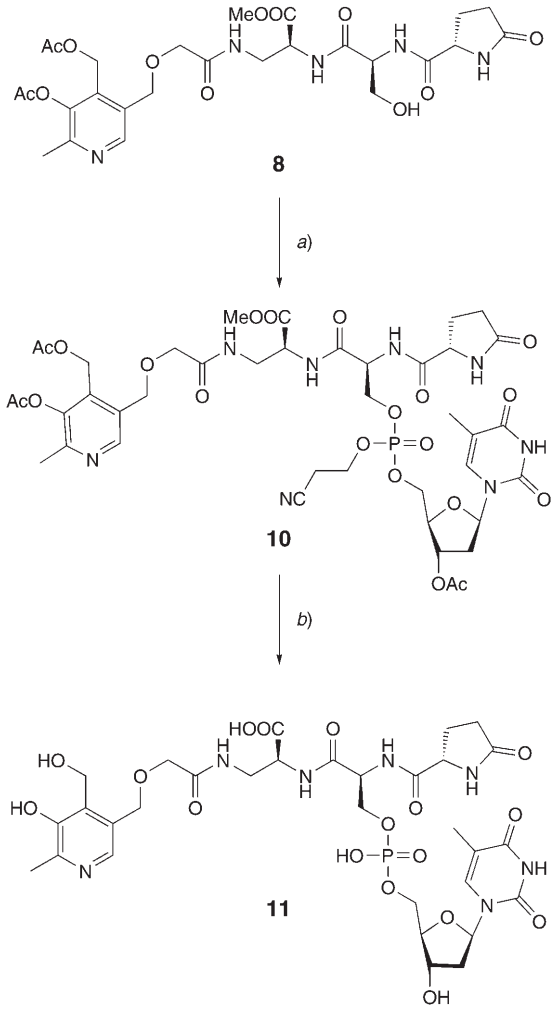
5a R = ^{t}BDMSi
5b R = ^{t}BDPSi

hydrogenation, and pyroglutamic acid was coupled to the free amino group using DCC and a catalytic amount of DMAP (*Scheme 2*). Here, we faced some problems. At the end of the synthesis, we would like to obtain the title compound, by a simple deprotection procedure with base. This means, however, that the isopropylidene group needs to be hydrolyzed and replaced by Ac protecting groups. Selective removal of the isopropylidene groups with acid, without loss of the ^{t}BuDMSi protecting group at the serine position of **6a** is, however, very difficult. Therefore, the ^{t}BDMSi group was removed using fluoride in MeOH and replaced by the more stable (*tert*-butyl)diphenylsilyl (^{t}BuDPSi) group, followed by selective removal of the isopropylidene protecting groups using pyridinium *para*-toluenesulfonate (PPTS). Both OH groups of the pyridoxine group were then acetylated with Ac_2O. The ^{t}BDPSi group can be selectively removed with fluoride in MeOH to afford the key compound **8**. It should be noted that we kept the diaminopropanoic acid linker as methyl ester during the whole synthetic procedure, as the more stable carboxamide congener is also more labile to racemization.

A new, more straightforward route is the protection of the primary OH group of serine with a ^{t}BDPSi group from the start of the reaction. This procedure avoids the deprotection–reprotection step using ^{t}BDMSi and ^{t}BDPSi. The key compound **8** can now be used for loading with the appropriate nucleotide following full deprotection. In a first instance, AZT was used, but the conditions of coupling and final global deprotection did not allow us to isolate the final compound (for which the reason is unknown). Therefore, we turned to thymidine as deliverable nucleotide. Thymidine is first converted to its 5′-phosphoramidite **9** (*Scheme 5*). This compound was coupled to the free OH group of the serine moiety of **8**, using 1*H*-tetrazole, followed by oxidation with I_2 in H_2O/pyridine.

Finally, treating of **10** with MeONa in MeOH resulted in the removal of the Ac as well as the 2-cyanoethyl protecting group. As the use of MeONa resulted in considerable loss of nucleotide *via* β-elimination, it was more appropriate to change the reaction conditions and use a 10% aqueous K_2CO_3 solution in MeOH to obtain the fully deprotected drug delivery system (*Scheme 5*). Incubation of compound **11** with a

Scheme 5



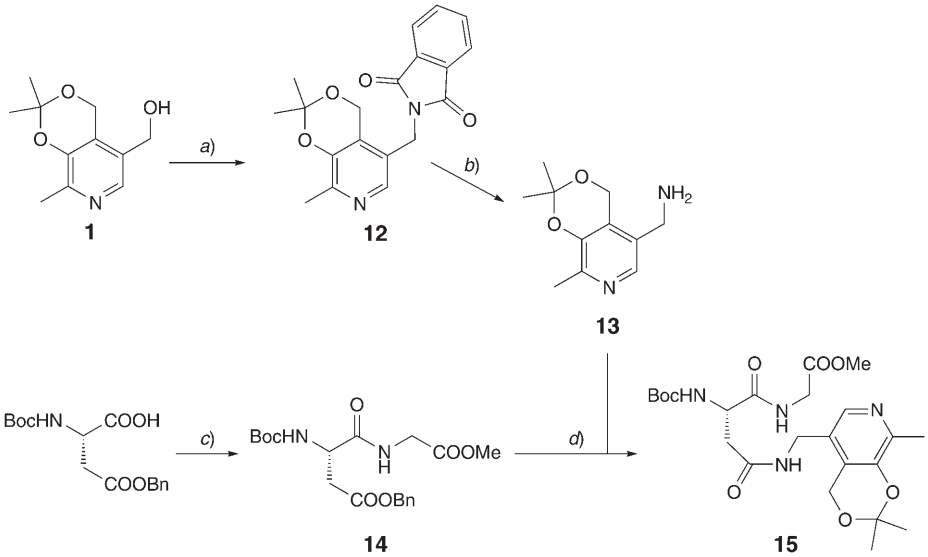
a) 1. 3′-*O*-Acetylthymidine, ($^{i}Pr_2N$)(Cl)P(CNE), Et_3N, MeCN (→ thymidine-5′-phosphoramidite (**9**)); 2. 1*H*-tetrazole, **8**, DMF; 3. I_2/Pyridine/H_2O. *b*) Aq. K_2CO_3 (10%), MeOH.

partially purified extract containing pyroglutamase demonstrates that the pyroglutamic acid (pGlu; end-cap of construct **11**) is easily removed in an enzymatic way so that the free terminal amino group is liberated. This reaction was followed by mass spectrometry.

In this first example (compound **11**), the pyridoxine moiety is situated at the end of the peptide chain. However, in case the peptide backbone would be the main recognition element for the delivery process, it could be advantageous to connect the pyridoxine at an internal position of the peptide chain (compound **20**; *cf. Scheme 7*).

For this reason, the dipeptide building block **15**, having a pyridoxine moiety in the side chain, was synthesized (*Scheme 6*).

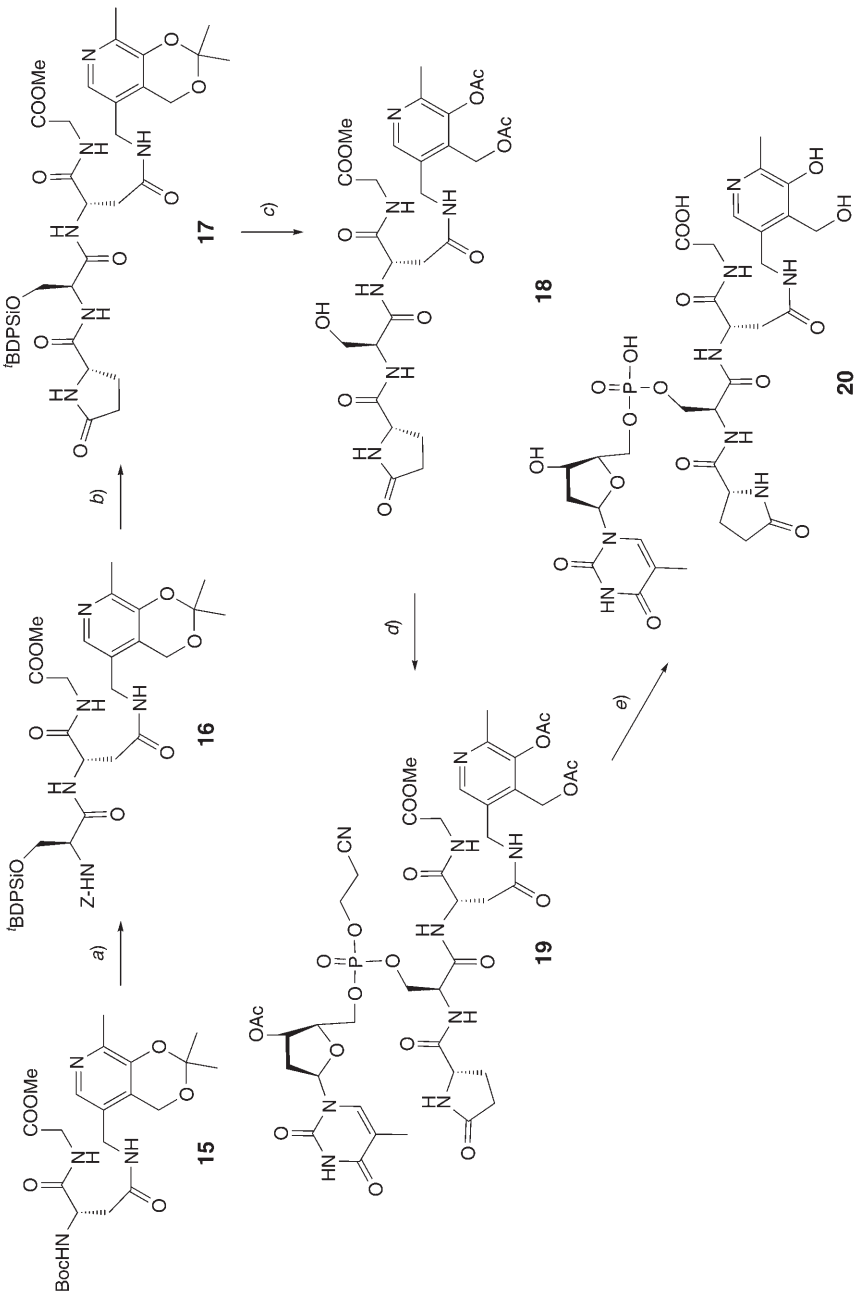
Scheme 6



a) Phthalimide, PPh_3, diazopropyl azodicarboxylate (DIAD), THF. *b*) Hydrazine, EtOH, reflux. *c*) HCl·GlyOMe, DCC, DMAP, CH_2Cl_2. *d*) 1. H_2/Pd–C (10%), AcOEt/MeOH 1:1; 2. **13**, DCC, DMAP, MeCN.

Aspartic acid, protected with a *tert*-butoxycarbonyl (Boc) group at the primary amino group and a benzyl (Bn) group at the β-carboxylic acid function, was reacted with the methyl ester of glycine to form **14** (*Scheme 6*). The Bn group in the side chain of the dipeptide was removed by hydrogenation, and the free carboxylic acid function was used to load the dipeptide with the amino derivative of pyridoxine **13**, which could be obtained from 3,4′-*O*-isopropylidenepyridoxine (**1**) by reacting with phtalimide to form **12** and deprotection with hydrazine (*Scheme 6*). Boc Deprotection of **15** and coupling with the ^{t}BDPSi-protected serine **5b** delivered **16** (*Scheme 7*). Removal of the Z group of **16** by hydrogenation, followed by condensation with pyroglutamic acid, gave the fully protected tetrapeptide **17**. The strategy that we developed for the synthesis of **8** worked for the synthesis of **18** as well. This means that the isopropylidene protecting group of **17** can be removed with acid, without deblocking of the hydroxyl group of the serine moiety (which is protected with a ^{t}BDPSi group). The free OH groups of the pyridoxine moiety were protected with Ac groups, followed by removal of the ^{t}BDPSi protecting group with fluoride. Reaction of the serine hydroxyl group of the protected peptide **18** with the phosphoramidite **9** of 3′-*O*-acetylthymidine, followed by oxidation, afforded the nucleotide–peptide conjugate **19**. All protecting groups could be removed simultaneously with K_2CO_3 in aqueous MeOH to obtain the highly functionalized delivery system **20** (*Scheme 7*). Further research will be focussed on the evaluation of compounds **11** and **20** for their potential to deliver thymidine 5′-monophosphate into cells.

Scheme 7



a) 1. CH_2Cl_2/CF_3COOH (TFA) 2:1, 0°; 2. **5b**, (Benzotriazol-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), Et_3N, DMF. *b*) 1. H_2/Pd–C (10%), AcOEt/MeOH 1:1; 2. Pyroglutamic acid, BOP, Et_3N, DMF. *c*) 1. Pyridinium *para*-toluenesulfonate (PPTS), EtOH, reflux; 2. Ac_2O, pyridine; 3. TBAF (1M), THF, 0°. *d*) 1. 3′-*O*-Acetylthymidine (**9**), ($^iPr_2N$)(Cl)P(CNE), Et_3N, MeCN (→thymidine-5′-phosphoramidite (**9**)); 2. 1*H*-tetrazole, DMF; 3. I_2/Pyridine/H_2O. *e*) Aq. K_2CO_3 (10%), MeOH.

Experimental Part

General. For all reactions, anal. grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware under N_2. Anh. THF and Et_2O were refluxed over Na/benzophenone and distilled. Pyridoxine and amino acids were provided by *Acros*, *Fluka*, and *Senn Chemicals*, and 2′-deoxythymidine by *Pharma Waldhof*. TLC: Precoated aluminium sheets (*Fluka* silica gel/TLC cards, 254 nm); the spots were visualized with UV light. Column chromatography (CC): *ICN* silica gel 63–200 60 Å. ^{1}H- and ^{13}C-NMR spectra: a *Varian Unity* 500-MHz spectrometer, a 200-MHz *Varian Gemini* apparatus, a *Bruker Avance* 300-MHz, or a *Bruker Avance* 500-MHz spectrometer. For sake of clarity, NMR signals of nucleoside-sugar-moiety H- and C-atoms are indicated with primes, signals of pyridoxyl moiety H- and C-substituent atoms are given with primes, and signals of peptide H- and C-atoms are indicated with *Greek* letters as positions in each amino acid. Exact mass measurements were performed on a quadrupole time-of-flight mass spectrometer (*Q-Tof-2*, *Micromass*, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface; samples were infused in i-PrOH/H_2O 1:1 at 3 µl/min.

3,4′-O-*Isopropylidenepyridoxine* (**1**). Conc. H_2SO_4 (14.57 ml, 273.4 mmol) was added to a stirred soln. of pyridoxine hydrochloride (50 g, 243 mmol) and 2,2-dimethoxypropane (249 ml, 2024 mmol) in dry CH_2Cl_2 (1000 ml). The soln. was refluxed for 8 h, and the org. layer was washed with a sat. soln. of $NaHCO_3$ and H_2O. The org. phase was dried (Na_2SO_4), filtered, and evaporated to dryness. The yellow solid was dissolved in a minimum of Et_2O, and petroleum ether was added dropwise to afford **1** (38.13 g, 75%). Light yellow precipitate. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.55 (*s*, *Me*$_2$C); 2.38 (*s*, Me–C(2)); 2.73 (br. *s*, OH); 4.56 (*s*, CH_2(4′)); 4.94 (*s*, CH_2(5′)); 7.84 (*s*, H–C(6)). ^{13}C-NMR ($CDCl_3$, 50 MHz): 18.27 (*Me*–C(2)); 24.68 (*Me*$_2$C); 58.52 (C(4′)); 60.28 (C(5′)); 99.79 (Me$_2$*C*); 125.89 (C(5)); 129.23 (C(4)); 138.76 (C(6)); 146.11 (C(2)); 147.96 (C(3)). HR-MS: 210.1111 ([*M*+H$^+$], $C_{11}H_{16}NO_3^+$; calc. 210.1131).

5′-O-*(Carboxymethyl)-3,4′*-O-*isopropylidenepyridoxine* (**2**). NaH (60% in mineral oil; 11.00 g, 275 mmol) was added to a stirred soln. of **1** (10.46 g, 50 mmol) and $ClCH_2COOH$ (9.45 g, 100 mmol) in dry THF (725 ml) at r.t. under N_2. The well-stirred mixture was refluxed for 12 h, and the suspension was cooled in an ice-bath. The pH was adjusted to 2–3 with 5N HCl, and the soln. was evaporated to dryness. The residue was subjected to silica-gel CC with CH_2Cl_2/MeOH 94:6 to afford **2**. The latter was dissolved in a mixture of CH_2Cl_2/MeOH 1:1, and Et_2O was added dropwise to obtain a white precipitate (10.47 g, 78%). ^{1}H-NMR (DMSO, 200 MHz): 1.49 (*s*, *Me*$_2$C); 2.29 (*s*, Me–C(2)); 4.04 (*s*, C*H*$_2$CO$_2$H); 4.48 (*s*, CH_2(5′)); 4.93 (*s*, CH_2(4′)); 7.92 (*s*, H–C(6)). ^{13}C-NMR (DMSO, 50 MHz): 18.45 (Me–C(2)); 24.61 (*Me*$_2$C); 58.11 (C(4′)); 66.98 (*C*H$_2$CO$_2$H); 67.61 (C(5′)); 99.76 (Me$_2$*C*); 126.44 (C(5)); 126.74 (C(4)); 139.70 (C(6)); 145.50 (C(2)); 146.86 (C(3)); 171.78 (CO$_2$H). HR-MS: 268.1180 ([*M*+H]$^+$, $C_{13}H_{18}NO_5^+$; calc. 268.1186).

Methyl 3-Amino-2-{[(benzyloxy)carbonyl]amino}propanoate Hydrochloride (**3**). $SOCl_2$ (9.87 ml, 136 mmol) was slowly added to a stirred suspension of Z-Dap-OH (Dap = 2,3-diaminopropanoic acid; 8.10 g, 34 mmol) in dry MeOH (272 ml) at 0° under N_2. The mixture was stirred at r.t. for 10 h, and the volatiles were removed under reduced pressure. The residue was co-evaporated with MeOH, and the white solid was dissolved in a minimum of MeOH. Et_2O was slowly added to afford **3** (9.35 g, 95%). White precipitate. ^{1}H-NMR (DMSO, 200 MHz): 3.00–3.26 (*m*, $CH_{2\beta}$); 3.68 (*s*, MeO); 4.45 (*dt*, *J* = 8.4, 4.8, CH_α); 5.07 (*s*, CH_2(Z)); 7.28–7.39 (*m*, 5 arom. H(Z)); 7.94 (*d*, *J* = 8.4, NH); 8.32 (br. *s*, NH_3). ^{13}C-NMR (DMSO, 50 MHz): 40.80 ($CH_{2\beta}$); 51.86 (CH_α); 52.65 (MeO); 66.01 (CH_2(Z)); 127.98–136.82 (6 arom. C(Z)); 156.30 (CONH); 170.05 (COO). HR-MS: 253.1181 ([*M*+H]$^+$, $C_{12}H_{17}N_2O_4$; calc. 253.1188).

5′-O-*{[*N*-(2-{[(benzyloxy)carbonyl]amino}-2-(methoxycarbonyl)ethyl)carbomoyl]methyl}-3,4′*-O-*isopropylidenepyridoxine* (**4**). 1,1′-Carbonyldiimidazole (CDI; 5.35 g, 33 mmol) was added to a stirred suspension of **2** (8.02 g, 30 mmol) in dry CH_2Cl_2 (90 ml) at 0° under N_2. The mixture was stirred at r.t. for 30 min (clear soln.), and it was cooled in an ice-bath. Dry Et_3N (4.58 ml, 33 mmol) was added, followed by **3** (9.09 g, 31.5 mmol), and the soln. was stirred at r.t. for 15 min. The mixture was diluted with CH_2Cl_2 (150 ml), and the org. layer was washed with H_2O and brine. The org. phase was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified on silica gel using a stepwise gradient of MeOH (0–2%) in CH_2Cl_2 to afford **4** (12.58 g, 83%). White foam. ^{1}H-NMR ($CDCl_3$, 300 MHz): 1.54 (*s*, *Me*$_2$C); 2.40 (*s*, Me–C(2)); 3.66–3.70 (*m*, $CH_{2\beta}$(Dap)); 3.74 (*s*, MeO); 3.90 (*s*, OC*H*$_2$CONH); 4.42 (br. *s*, CH_2(5′),

CH_α(Dap)); 4.84 (*s*, CH_2(4′)); 5.09 (*s*, CH_2(Z)); 5.88 (br. *s*, NH(Z)); 6.93 (br. *s*, NH(Dap)); 7.33 (*m*, 5 arom. H (Z)); 7.94 (*s*, H−C(6)). ^{13}C-NMR ($CDCl_3$, 75 MHz): 18.50 (*Me*−C(2)); 24.67, 24.73 (Me_2C); 40.82 ($CH_{2\beta}$(Dap)); 52.75 (MeO); 54.35 (CH_α(Dap)); 58.32 (C(4′)); 67.11 (CH_2(Z)); 68.58 (C(5′)); 69.27 (*C*H_2CONH); 99.88 (Me_2*C*); 124.88 (C(5)); 125.70 (C(4)); 128.04–136.30 (6 arom. C(Z)); 139.75 (C(6)); 146.08 (C(2)); 148.90 (C(3)); 156.20 (CO(Z)); 169.92 (*C*O_2Me); 170.59 (CONH(Dap)). HR-MS: 502.2189 ($[M+H]^+$, $C_{25}H_{32}N_3O_8^+$; calc. 502.2190).

Z-Ser(O^t*BDPSi)-OH* (**5b**). ^{t}BDPSiCl (20.80 ml, 80 mmol) was added to a stirred soln. of Z-serine (4.78 g, 20 mmol) and 1*H*-imidazole (10.89 g, 160 mmol) in dry DMF (40 ml) at 60° under N_2. The mixture was stirred at 60° for 16 h, and AcOEt was added (150 ml). The org. layer was washed with brine, and the volatiles were removed. The residue was suspended in THF/H_2O 1:1 (60 ml), and solid K_2CO_3 (0.28 g, 2.00 mmol) was added. The soln. was stirred 1 h, and the THF was evaporated under reduced pressure. The pH was adjusted to 2–3 with 1N HCl, and the aq. layer was extracted with AcOEt. The combined org. phases were collected, dried (Na_2SO_4), filtered, and evaporated. The residue was subjected to silica-gel CC with hexane/AcOEt 8:2 to afford **5b** (8.50 g, 89%), which could be crystallized from hexane. ^{1}H-NMR ($CDCl_3$, 500 MHz): 1.01 (*s*, Me_3CSi); 3.92 (*dd*, *J* = 10.0, 3.8, C*H*H_β); 4.15 (br. *d*, *J* = 10.0, CH*H*$_\beta$); 4.45–4.49 (*m*, CH_α); 5.12 (*s*, CH_2(Z)); 5.60 (*d*, *J* = 7.8, NH); 7.32–7.62 (*m*, 15 arom. H(^{t}BDPSi) and arom. H(Z)); 8.35 (br. *s*, COOH). ^{13}C-NMR ($CDCl_3$, 125 MHz): 19.97 (Me_3*C*Si); 26.72 (*Me*$_3$CSi); 55.77 (CH_α); 64.20 ($CH_{2\beta}$); 67.15 (CH_2(Z)); 127.83–135.46 (6 arom. C(Z)); 128.10–135.54 (12 arom. C(^{t}BDPSi)); 156.10 (CONH); 175.17 (COOH). HR-MS: 478.2055 ($[M+H]^+$, $C_{27}H_{32}NO_5Si^+$; calc. 478.2049).

Z-Ser(O^t*BDPSi)-Dap(3,4′*-O-*isopropylidenepyridoxin-5′*-O-*acetyl)-OMe* (**6b**). Pd/C (1.24 g, 10% in weight) was added to a stirred soln. of **4** (12.39 g, 24.7 mmol) in MeOH (125 ml) at r.t. The flask was purged several times, and an atmosphere of H_2 was maintained for 45 min. The catalyst was filtered off over a paper filter, washed several times with a mixture of CH_2Cl_2/MeOH 1:1, and the solvents were evaporated to dryness to afford the free amine as white foam (9.32 g). The latter was dissolved in dry CH_2Cl_2 (120 ml), and the soln. was stirred at 0° under N_2. Then, DCC (5.11 g, 24.79 mmol) and compound **5b** (10.75 g, 22.54 mmol) were added, followed by the addition of a cat. amount of DMAP. The soln. was stirred at r.t. for 15 min, and the dicyclohexylurea (DCU) was filtered off. The solvent was removed under reduced pressure, and the residue was subjected to silica-gel CC with a stepwise gradient of MeOH (0–2%) in CH_2Cl_2 to afford **6b** (16.94 g, 91% based on compound **4**). White foam. ^{1}H-NMR ($CDCl_3$, 500 MHz): 1.03 (*s*, Me_3CSi); 1.55 (*s*, Me_2C); 2.40 (*s*, Me−C(2)); 3.65–3.72 (*m*, $CH_{2\beta}$(Dap), C*H*H_β(Ser)); 3.74 (*s*, MeO); 3.90 (*s*, OC*H*$_2$CONH); 4.01 (*dd*, *J* = 9.8, 3.9, CH*H*$_\beta$(Ser)); 4.23–4.25 (*m*, CH_α(Dap)); 4.44 (*s*, CH_2(5′)); 4.58–4.62 (*m*, CH_α(Ser)); 4.86 (*s*, CH_2(4′)); 5.11 (*s*, CH_2(Z)); 5.80 (br. *s*, NH(Z)); 6.98 (*t*, *J* = 6.0; NH_β(Dap)); 7.32–7.62 (*m*, 15 arom. H(^{t}BDPSi), arom. H(Z)); 7.45 (*d*, *J* = 6.8, NH(Ser)); 7.96 (*s*, H−C(6)). ^{13}C-NMR ($CDCl_3$, 125 MHz): 18.50 (Me_3*C*Si); 19.15 (*Me*−C(2)); 24.66 (*Me*$_2$C); 26.72 (*Me*$_3$CSi); 40.72 ($CH_{2\beta}$(Dap)); 52.76 (MeO); 53.21 (CH_α(Dap)); 56.60 (CH_α(Ser)); 58.31 (C(4′)); 63.79 ($CH_{2\beta}$(Ser)); 67.31 (CH_2(Z)); 68.50 (C(5′)); 69.14 (*C*H_2CONH); 99.83 (Me_2*C*); 124.88 (C(5)); 125.67 (C(4)); 127.83–135.46 (6 arom. C(Z)); 128.10–135.54 (12 arom. C(^{t}BDPSi)); 139.72 (C(6)); 146.04 (C(2)); 148.83 (C(3)); 156.17 (CO(Z)); 169.90 (*C*O_2Me); 170.06 (CONH(Ser)); 170.36 (CONH(Dap)). HR-MS: 827.3679 ($[M+H]^+$, $C_{44}H_{55}N_4O_{10}Si^+$; calc. 827.3687).

pGlu-Ser(O^t*BDPSi)-Dap(3,4′*-O-*isopropylidenepyridoxin-5′*-O-*acetyl)-OMe* (**7b**). Pd/C (1.28 g, 10% in weight) was added to a stirred soln. of **6b** (12.84 g, 15.55 mmol) in MeOH (110 ml) at r.t. The flask was purged several times, and an atmosphere of H_2 was maintained for 45 min. The catalyst was filtered off over a paper filter, washed several times with a mixture of CH_2Cl_2/MeOH 1:1, and the solvents were evaporated to dryness to afford the free amine as white foam (10.41 g, 15.05 mmol). The latter was dissolved in dry MeCN (150 ml), and the soln. was stirred at 0° under N_2. Then, DCC (4.97 g, 24.08 mmol) and pyroglutamic acid (pGlu; 3.20 g, 24.83 mmol) were added, followed by the addition of a cat. amount of DMAP. The soln. was stirred at r.t. for 30 min, and the DCU was filtered off. The solvent was removed under reduced pressure, and the residue was subjected to silica-gel CC with CH_2Cl_2/MeOH 96:4 to afford **7b** (8.34 g, 69%). White foam. ^{1}H-NMR ($CDCl_3$, 500 MHz): 1.03 (*s*, Me_3CSi); 1.54 (*s*, Me_2C); 1.89–1.95 (*m*, C*H*$H_{2\beta}$(pGlu)); 2.03–2.17 (*m*, $CH_{2\gamma}$(pGlu)); 2.20–2.28 (*m*, CH*H*$_{2\beta}$(pGlu)); 2.30 (*s*, Me−C(2)); 3.41–3.52 (*m*, $CH_{2\beta}$(Dap)); 3.60 (br. *s*, $CH_{2\beta}$(Ser), MeO); 3.89 (*s*, OC*H*$_2$CONH); 4.10 (*dd*, *J* = 8.8, 4.4, CH_α(pGlu)); 4.32 (*m*, CH_α(Ser)); 4.38 (*dd*, *J* = 13.2, 6.8, CH_α(Dap)); 4.47 (*s*, CH_2(5′));

4.90 (br. *s*, CH_2(4′)); 7.28–7.56 (*m*, 10 arom. H(^{t}BDPSi)); 7.77 (br. *s*, H–C(6)); 7.94–7.98 (*m*, NH_β(Dap), NH_α(Dap), NH(pGlu)); 8.23 (*d*, *J* = 7.3, NH(Ser)). ^{13}C-NMR ($CDCl_3$, 125 MHz): 18.39 (Me_3CSi); 19.16 (Me–C(2)); 24.65 (Me_2C); 25.80 ($CH_{2\beta}$(pGlu)); 26.69 (Me_3CSi); 29.41 ($CH_{2\gamma}$(pGlu)); 40.92 ($CH_{2\beta}$(Dap)); 52.69 (MeO); 54.09 (CH_α(Dap)); 54.65 (CH_α(Ser)); 57.21 (CH_α(pGlu)); 58.21 (C(4′)); 63.43 ($CH_{2\beta}$(Ser)); 68.42 (C(5′)); 69.17 (CH_2CONH); 99.89 (Me_2C); 127.81 (C(5)); 127.89 (C(4)); 124.77–135.46 (12 arom. C(^{t}BDPSi)); 139.52 (C(6)); 146.03 (C(2)); 148.85 (C(3)); 169.65 (CO_2Me); 169.91 (CONH(Ser)); 172.02 (CONH(Dap)); 172.46 (CO_δ(pGlu)); 178.52 (CONH(pGlu)). HR-MS: 804.3643 ($[M+H]^+$, $C_{41}H_{54}N_5O_{10}Si^+$; calc. 804.3640).

*pGlu-Ser-Dap(3,4′-*O*-diacetylpyridoxin-5′-*O*-acetyl)-OMe* (**8**). To a soln. of **7b** (7.2 g, 9 mmol) in abs. EtOH (90 ml) was added pyridinium *para*-toluenesulfonate (PPTS) (5.65 g, 22.25 mmol), and the mixture was refluxed for 3 d. After cooling, the soln. was neutralized by addition of Na_2CO_3, and the solvent was evaporated under reduced pressure. The white solid was partitioned between AcOEt and H_2O, and the org. layer was decanted and washed with brine. It was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by silica-gel CC with CH_2Cl_2/MeOH 92 : 8 to afford the deprotected compound (4.40 g, 64%). White solid.

Ac_2O (1.61 ml, 17.1 mmol) was added to a stirred soln. of deprotected compound (4.40 g, 5.70 mmol) in dry pyridine (60 ml) at r.t. under N_2. After 14 h, the pyridine was removed under reduced pressure, and the residue was dissolved in AcOEt, and the org. layer was washed with a 5% soln. of Na_2CO_3 and H_2O, and dried (Na_2SO_4). After filtration, the solvent was evaporated to dryness, and the residue was co-evaporated with toluene and MeOH. The crude white solid was used for next reaction without further purification.

The white crude material was dissolved in THF (57 ml) at 0°, and a soln. of Bu_4NF (1M in THF; 5.7 ml, 5.70 mmol) was added dropwise. Then, the mixture was stirred at r.t. for 20 min. The solvent was evaporated under reduced pressure, and the residue was subjected to silica-gel CC with a stepwise gradient of MeOH (10–12%) in CH_2Cl_2 to afford **8**, which was recrystallized from MeOH (2.11 g, 61%). ^{1}H-NMR (DMSO, 500 MHz): 1.89–1.95 (*m*, 1 H, $CH_{2\beta}$(pGlu)); 1.97 (*s*, Ac); 2.03–2.28 (*m*, CH_γ(pGlu), 1 H of $CH_{2\beta}$(pGlu)); 2.31 (*s*, Me–C(2)); 2.36 (*s*, Ac); 3.40–3.52 (*m*, $CH_{2\beta}$(Dap)); 3.60 (br. *s*, $CH_{2\beta}$(Ser), MeO); 3.94 (*s*, OCH_2CONH); 4.09–4.11 (*m*, CH_α(pGlu)); 4.30–4.31 (*m*, CH_α(Ser)); 4.38 (*dd*, *J* = 13.3, 6.7, CH_α(Dap)); 4.68 (*s*, CH_2(5′)); 4.91 (*t*, *J* = 5.6, OH(Ser)); 5.10 (*s*, CH_2(4′)); 7.78 (*s*, H–C(6)); 7.98 (*m*, NH_α(Dap), NH(pGlu)); 8.24–8.28 (br. *s*, NH_β(Dap)); 8.44 (*d*, *J* = 7.2, NH(Ser)). ^{13}C-NMR ($CDCl_3$, 125 MHz): 19.28 (Me–C(2)); 20.42 (2 *Me*CO); 25.22 ($CH_{2\beta}$(pGlu)); 29.30 ($CH_{2\gamma}$(pGlu)); 40.20 ($CH_{2\beta}$(Dap)); 52.15 (MeO); 52.40 (CH_α(Dap)); 55.11 (CH_α(Ser)); 55.71 (CH_α(pGlu)); 56.84 (C(4′)); 61.58 ($CH_{2\beta}$(Ser)); 67.61 (C(5′)); 69.33 (CH_2CONH); 130.99 (C(5)); 135.63 (C(4)); 144.35 (C(6)); 147.04 (C(2)); 152.04 (C(3)); 168.83 (Me*C*O); 169.77 (CO_2Me); 170.07 (CONH(Ser)); 170.12 (Me*C*O); 170.68 (CONH(Dap)); 172.64 (CO_δ(pGlu)); 177.65 (CO(pGlu)). HR-MS: 610.2365 ($[M+H]^+$, $C_{26}H_{35}N_5O_{12}^+$; calc. 610.2361).

*3′-*O*-Acetylthymidine*. To a soln. of thymidine (1.5 g, 6.5 mmol) in dry pyridine (40 ml) at 0° under N_2 was added dimethoxytrityl chloride (2.43 g, 7.15 mmol), and the mixture was stirred at r.t. overnight. The solvent was evaporated under reduced pressure and co-evaporated with toluene, and the crude oil was purified by silica-gel CC with hexane/AcOEt/Et_3N 1 : 9 : 0.5. Then, the 3′-OH group was protected with Ac_2O (1.5 ml) in dry pyridine (25 ml) at r.t. overnight. The volatiles were removed under reduced pressure, and the remaining oil was dissolved in CH_2Cl_2/MeOH 9 : 1 (65 ml), and TFA (0.650 ml) was added dropwise. After a few min, the volatiles were removed under reduced pressure, and the remaining product was purified on silica-gel CC with hexane/AcOEt 2 : 8 as eluent to afford the title compound (1.1 g, 60%). White solid. ^{1}H-NMR ($CDCl_3$, 300 MHz): 1.77 (*s*, Me–C(5)); 2.06 (*s*, *Me*CO); 2.23–2.26 (*m*, CH_2(2′)); 3.61–3.64 (*m*, CH_2(5′)); 3.96–3.98 (*m*, H–C(4′)); 5.18–5.22 (*m*, H–C(3′), OH); 6.17 (*t*, *J* = 7, H–C(1′)); 7.72 (*s*, H–C(6)); 11.31 (*s*, NH). ^{13}C-NMR (DMSO, 75 MHz): 12.72 (*Me*–C(5)); 21.29 (*Me*CO); 36.94 (C(2′)); 61.78 (C(5′)); 75.14 (C(3′)); 84.12 (C(4′)); 85.02 (C(1′)); 110.17 (C(5)); 136.26 (C(6)); 150.93 (C(2)); 164.11 (C(4)); 170.47 (Me*C*O). HR-MS: 285.1082 ($[M+H]^+$, $C_{12}H_{16}N_2O_6^+$; calc. 285.1086).

*pGlu-Ser[*O*-(2-cyanoethyl)thymidinephosphotriester]-Dap(3,4′-*O*-diacetylpyridoxin-5′-*O*-acetyl)-OMe* (**10**). 2-Cyanoethyl chloro(diisopropyl)phosphoramidite (0.154 ml, 0.689 mmol) was added to a stirred soln. of Et_3N (0.111 ml, 0.787 mmol) and 3′-*O*-acetylthymidine (0.168 g, 0.590 mmol) in dry

MeCN (2.5 ml) at 0° under N_2 (→thymidine-5′-phosphoramidite (**9**)). The mixture was stirred at r.t. for 20 min and was cooled in an ice bath. Then, a 0.45M solution of 1*H*-tetrazole in dry MeCN (4.4 ml) was added, followed by the addition of compound **8** (0.280 g, 0.492 mmol) in dry DMF (1.5 ml). The mixture was stirred at r.t. for 2 h, and a 0.02M oxidative standard soln. (I_2/H_2O/pyridine, 30 ml) was added. After 30 min stirring, the mixture was diluted with CH_2Cl_2 (30 ml), and the org. layer was washed with a 20% hydrogen sulfite soln. and brine. It was dried (Na_2SO_4) and evaporated to dryness. The residue was subjected to silica-gel CC with CH_2Cl_2/MeOH 9:1 to afford **10** (0.284 g, 61%). White solid. ^{1}H-NMR (DMSO, 300 MHz): Pyridoxin-5′-*O*-acetyl: 1.97 (*s*, *Me*CO); 2.31 (*s*, Me); 2.36 (*s*, *Me*CO); 3.94 (*s*, CH_2CO); 4.68 (*s*, CH_2O); 5.09 (*s*, C*H*$_2$OAc); 7.51 (*s*, H−C(6)); Dap: 3.40, 3.49 (2 br. *s*, $CH_{2\beta}$); 4.05 (br. *s*, CH_α); 8.00 (*s*, NH_β); 8.43 (*s*, NH_α); Ser: 3.43–3.51 (*m*, $CH_{2\beta}$); 4.68 (br. *s*, CH_α); 8.53 (*s*, NH); pGlu: 2.04–2.23 (*m*, $CH_{2\beta}$, $CH_{2\gamma}$); 4.39 (*dd*, *J* = 8.7, 4.5, CH_α); 8.34 (*s*, NH); Thy: 1.78 (*s*, Me); 2.04, 2.12 (*m*, CH_2(2′)); 2.07 (*s*, *Me*CO); 2.90 (br. *s*, CH_2CN); 4.05–4.09 (*m*, CH_2(5′)); 4.15–4.18 (*m*, H−C(4′)); 4.22 (br. *s*, CH_2OP); 5.19 (br. *s*, H−C(3′)); 6.20 (*t*, *J* = 7, H−C(1′)); 7.72 (*s*, H−C(6)); 11.38 (*s*, NH). ^{13}C-NMR ($CDCl_3$, 75 MHz): Pyridoxin-5′-*O*-acetyl: 19.39 (Me); 19.63 (*Me*CO); 20.77 (*Me*CO); 67.09 (*C*H$_2$OAc); 81.96 (CH_2O); 82.05 (*C*H$_2$CO); 131.31 (C(5)); 136.00 (C(4)); 144.69 (C(6)); 147.36 (C(2)); 150.87 (C(3)); 170.38 (Me*C*O); 170.50 (CH_2*C*O); 170.81 (Me*C*O); Dap: 52.54 (C_β); 56.08 (MeO); 57.17 (C_α); 170.18 (CO); Ser: 52.87 (C_α); 67.94 (C_β); 168.60 (CO); pGlu: 25.55 (C_β); 29.56 (C_γ); 63.04 (C_α); 173.25 (CO_δ); 177.87 (CO); Thy: 12.50 (Me); 19.51 (*C*H$_2$CN); 21.20 (*Me*CO); 36.04 (C(2′)); 67.51 (CH_2OP); 69.67 (C(5′)); 84.43 (C(3′)); 83.86 (C(1′)); 85.9 (C(4′)); 110.61 (C(5)); 118.62 (CH_2*C*N); 135.92 (C(6)); 152.36 (C(2)); 164.08 (C(4)); 169.14 (Me*C*O). HR-MS: 1009.3196 ($[M+H]^+$, $C_{41}H_{54}N_8O_{20}P^+$; calc. 1009.3192.

*pGlu-Ser(*O*-thymidinephosphodiester)-Dap(pyridoxin-5′-*O*-acetyl)-OH* (**11**). Compound **10** (0.208 g, 0.206 mmol) was dissolved in MeOH (10 ml), and the mixture was cooled at 0°. Then, a soln. of K_2CO_3 10% (10 ml) was added dropwise, and the mixture was stirred overnight (the temp. was raised slowly to r.t.). The pH was then adjusted to 3 with *DOWEX* H^+, and the solvent was removed. The residue was purified on silica gel with $CHCl_3$/MeOH/H_2O 5:4:1 to afford **11** which was lyophilized from H_2O (0.048 g, 29%). ^{1}H-NMR (DMSO, 500 MHz): Pyridoxin-5′-*O*-acetyl: 2.32 (*s*, Me); 3.86 (*s*, CH_2CO); 4.50 (*s*, CH_2O); 4.81 (*s*, C*H*$_2$OH); 5.51 (br. *s*, CH_2O*H*); 7.86 (*s*, H−C(6), OH); Dap: 3.22, 3.39 (2 br. *s*, $CH_{2\beta}$); 3.94 (br. *s*, CH_α); 7.75 (*s*, NH_β); 8.07 (*s*, NH_α); Ser: 3.85–3.94 (*m*, $CH_{2\beta}$); 4.34 (br. *s*, CH_α); 8.97 (*s*, NH); pGlu: 2.04–2.23 (*m*, $CH_{2\beta}$, $CH_{2\gamma}$); 4.08 (*dd*, *J* = 8.9, 4.3, CH_α); 7.86 (*s*, NH); Thy: 1.78 (*s*, Me); 2.04, 2.12 (*m*, CH_2(2′)); 3.86 (*m*, CH_2(5′)); 3.86–3.88 (*m*, H−C(4′)); 4.28 (br. *s*, H−C(3′)); 5.51 (br. *s*, OH); 6.20 (*t*, *J* = 7, H−C(1′)); 7.74 (*s*, H−C(6)); 11.26 (*s*, NH). ^{13}C-NMR ($CDCl_3$, 125 MHz): Pyridoxin-5′-*O*-acetyl: 19.27 (Me); 57.29 (CH_2OH); 68.17 (CH_2O); 69.16 (CH_2CO); 128.49 (C(5)), 132.25 (C(4)); 140.00 (C(6)); 147.26 (C(2)); 150.65 (C(3)); 172.71 (CO); Dap: 41.28 (C_β); 53.36 (C_α); 177.68 (COOH); Ser: 53.56 (C_α); 64.19 (C_β); 168.90 (CO); pGlu: 25.32 (C_β); 29.34 (C_γ); 55.68 (C_α); 172.11 (CO_δ); 177.70 (CO): Thy: 12.16 (Me); 39.55 (C(2′)); 64.94 (C(5′)); 71.04 (C(3′)); 83.86 (C(1′)); 85.9 (C(4′)); 109.95 (C(5)); 136.25 (C(6)); 150.65 (C(2)); 163.92 (C(4)). HR-MS: 814.2275 ($[M-H]^-$, $C_{31}H_{41}N_7O_{17}P^-$; calc. 814.2296).

*3,4′-*O*-Isopropylidene-2-methyl-5-(phthalimidomethyl)pyridine* (**12**). To a soln. of **1** (20.9 g, 100 mmol), Ph_3P (39.3 g, 150 mmol) and phthalimide (22 g, 150 mmol) in dry THF (750 ml) under N_2 was added dropwise a soln. of diisopropylazodicarboxylate (DIAD; 30 ml; 150 mmol) in dry THF (150 ml) for 2 h. The solvent was removed under reduced pressure, and the residue was purified by silica-gel CC with hexane/AcOEt 6:4 as eluent to afford **12** (30.4 g, 90%). Light yellow solid. ^{1}H-NMR (DMSO, 300 MHz): 1.46 (*s*, Me_2C); 2.29 (*s*, Me−C(2)); 4.68 (*s*, CH_2(4′)); 5.06 (*s*, CH_2N); 7.84–7.96 (*m*, 4 arom. H); 7.95 (*s*, H−C(6)). ^{13}C-NMR (DMSO, 75 MHz): 18.66 (*Me*−C(2)); 24.95 (*Me*$_2$C); 35.58 (CH_2N); 58.50 (C(4′)); 99.97 (Me_2*C*); 123.66 (2 arom. C (phthalimido)); 125.89 (C(5)); 132.02 (C(4)); 134.97 (4 arom. C (phthalimido)); 140.21 (C(6)); 145.46 (C(2)); 146.21 (C(3)); 168.16 (2 C=O). HR-MS: 339.1336 ($[M+H]^+$, $C_{19}H_{19}N_2O_4^+$; calc. 339.1344).

*5-(Aminomethyl)-3,4′-*O*-isopropylidene-2-methylpyridine* **13**. Compound **12** (7 g, 20.7 mmol) was suspended in EtOH (100 ml) and $NH_2NH_2 \cdot H_2O$ (2 ml, 41.4 mmol) was added. The well-stirred mixture was refluxed for 45 min, then cooled, and a KOH soln. (2N, 270 ml) was added. The product was extracted several times with $CHCl_3$, and the org. layers were collected, washed with brine, dried (Na_2SO_4), filtered, and evaporated to dryness. The resulting oil was dissolved in Et_2O, and the soln. was heated a few min and put into dry ice for 20 min to give a white precipitate. The solid was filtered, and the filtrate was

concentrated, and the same procedure was repeated twice to afford **13** (3.55 g, 83%). ^{1}H-NMR (DMSO, 300 MHz): 1.46 (*s*, *Me*$_2$C); 2.25 (*s*, Me–C(2)); 2.52 (br. *s*, NH$_2$); 3.58 (*s*, CH$_2$N); 4.89 (*s*, CH$_2$(4′)); 7.91 (*s*, H–C(6)). ^{13}C-NMR (DMSO, 75 MHz): 18.62 (Me–C(2)); 24.95 (*Me*$_2$C); 40.33 (CH$_2$N); 58.33 (C(4′)); 99.64 (Me$_2$*C*); 125.44 (C(5)); 132.56 (C(4)); 139.20 (C(6)); 145.01 (C(2)); 145.39 (C(3)). HR-MS: 209.1283 ([*M*+H]$^+$, $C_{11}H_{17}N_2O_2^+$; calc. 209.1290).

Boc-Asp(OBn)-Gly-OMe (**14**). Into a stirred suspension of HCl·Gly-OMe (5.52 g, 44 mmol) in dry CH$_2$Cl$_2$ (50 ml), NH$_3$ was bubbled through for 5 min. The solvent was removed under reduced pressure without heating and suspended again in dry CH$_2$Cl$_2$ (200 ml). The mixture was cooled at 0°, and Boc-Asp(OBn)-OH (12.9 g, 40 mmol), DCC (9 g, 44 mmol), and DMAP (488 mg, 4 mmol) were successively added. The mixture was stirred at r.t. for 1 h, filtered, and washed with CH$_2$Cl$_2$. The filtrate and the washings were collected, and evaporated to dryness. The residue was purified by silica-gel CC with hexane/AcOEt 6:4 to afford **14** (11.8 g, 98%). White solid. ^{1}H-NMR (DMSO, 300 MHz): 1.37 (*s*, *t*-Bu); 2.46–2.84 (*m*, CH$_{2\beta}$(Asp)); 3.60 (*s*, MeO); 3.78–3.90 (*m*, CH$_{2\alpha}$(Gly)); 4.36–4.49 (*m*, CH$_\alpha$(Asp)); 5.08 (*s*, CH$_2$(Z)); 7.13–7.18 (*m*, NH(Asp)); 7.35 (*s*, 5 arom. H(Z)); 8.22 (br. *s*, NH(Gly)). ^{13}C-NMR (DMSO, 75 MHz): 28.60 (*Me*$_3$C); 36.79 (C$_\beta$(Asp)); 41.19 (C$_\alpha$(Gly)); 51.21 (C$_\alpha$(Asp)); 52.12 (MeO); 66.08 (CH$_2$(Z)); 78.62 (Me$_3$*C*); 128.23–136.51 (6 arom. C); 155.62 (CO(Boc)); 170.55 (CO$_2$(Gly)+CO$_\gamma$(Asp)); 171.75 (CO(Asp)). HR-MS: 359.1820 ([*M*+H]$^+$, $C_{19}H_{27}N_2O_7^+$; calc. 359.1818).

*Boc-Asp[(3,4′-*O*-isopropylidene-2-methylpyridin-5-yl)methyl]-Gly-OMe* (**15**). Pd/C (10%; 700 mg, 10% by weight) was added to a stirred solution of **14** (7 g, 17.76 mmol) in a degased mixture MeOH/AcOEt 1:1 (90 ml) under N$_2$ at r.t. The flask was purged several times with H$_2$, and an atmosphere of H$_2$, was maintained for 1 h (TLC: hexane/AcOEt 6:4). The catalyst was filtered off over *Celite*, washed several times with CH$_2$Cl$_2$/MeOH 1:1, and the solvents were evaporated to dryness to afford the free carboxylic acid as a white solid (5.3 g). The latter was dissolved in dry MeCN (175 ml), and the soln. was stirred at 0° under N$_2$. Then DCC (3.8 g, 19.2 mmol), **13** (4 g, 19.2 mmol), and a cat. amount of DMAP (427 mg; 1.8 mmol) were added successively. The mixture was vigorously stirred at r.t. for 2 h. The DCU was filtered off, the solvent was removed under reduced pressure, and the residue was subjected to silica-gel CC with CH$_2$Cl$_2$/MeOH 95:5 to afford **15** (6.1 g, 70%). White solid. ^{1}H-NMR (DMSO, 300 MHz): 1.37 (*s*, *t*-Bu); 1.48 (*s*, *Me*$_2$C); 2.26 (*s*, Me–C(2)); 2.36–2.49 (*m*, CH$_{2\beta}$(Asp)); 3.60 (*s*, MeO); 3.72–3.90 (*m*, CH$_{2\alpha}$(Gly)); 4.05–4.21 (*m*, C(5)–CH$_2$); 4.29–4.39 (*m*, CH$_\alpha$(Asp)); 4.83 (*s*, CH$_2$(4′)); 6.92 (*d*, *J* = 8.1, NH(Asp)); 7.88 (*s*, H–C(6)); 8.10–8.21 (*m*, NH(Gly), NH(Asp)). ^{13}C-NMR (DMSO, 75 MHz): 18.69 (Me–C(2)); 25.00 (*Me*$_2$C); 28.58 (*Me*$_3$C); 37.28 (C$_\beta$(Asp)); 37.87 (C(5)–*C*H$_2$); 41.17 (C$_\alpha$(Gly)); 51.58 (C$_\alpha$(Asp)); 52.10 (MeO); 58.33 (C(4′)); 78.71 (Me$_3$*C*); 99.86 (Me$_2$*C*); 125.79 (C(5)); 128.05 (C(4)); 139.98 (C(6)); 145.47 (C(2)); 145.70 (C(3)); 155.52 (CO(Boc)); 169.65 (CO$_\gamma$(Asp)); 170.60 (CO(Gly)); 172.44 (CO(Asp)). HR-MS: 495.2450 ([*M*+H]$^+$, $C_{23}H_{35}N_4O_8^+$; calc. 495.2455).

Z-Ser(O^t*BDPSi)-Asp[(3,4′-*O*-isopropylidene-2-methylpyridin-5-yl)methyl]-Gly-OMe* (**16**). The Boc protecting group of **15** (2.4 g, 4.86 mmol) was cleaved with CH$_2$Cl$_2$/CF$_3$COOH 2:1 (75 ml) at 0°. The acid mixture was stirred for 30 min at r.t. The solvents were evaporated *in vacuo*, and the remaining material was co-evaporated twice with cyclohexane. Then, the residue was dissolved in DMF (25 ml), and Z-Ser(O^{t}BDPSi)-OH (**5b**; 2.54 g, 5.34 mmol) was added, followed by BOP (2.36 g, 5.34 mmol). The pH of the mixture was adjusted by addition of Et$_3$N to 8–9. When no starting material was detected by TLC, the solvent was evaporated *in vacuo*, and the crude material was dissolved in AcOEt (100 ml). The org. layer was washed with KHSO$_4$ solution (1N), H$_2$O, sat. NaHCO$_3$ soln., and brine, then dried (Na$_2$SO$_4$), filtered, and concentrated under reduced pressure. The residue was absorbed on silica and purified by silica-gel CC with CH$_2$Cl$_2$/MeOH 95:5 to afford **16** (3.31 g, 80%). White solid. ^{1}H-NMR (DMSO, 300 MHz): 0.94 (*s*, *t*-Bu); 1.47 (*s*, *Me*$_2$C); 2.25 (*s*, Me–C(2)); 2.36–2.49 (*m*, CH$_{2\beta}$(Asp)); 3.56 (*s*, MeO); 3.68–3.93 (*m*, CH$_{2\alpha}$(Gly), CH$_{2\beta}$(Ser)); 3.99–4.20 (*m*, CH$_2$–C(5)); 4.30–4.41 (*m*, CH$_\alpha$(Asp)); 4.62–4.76 (*m*, CH$_\alpha$(Ser)); 4.81 (*s*, CH$_2$(4′)); 4.99–5.13 (*s*, CH$_2$(Z)); 7.26–7.66 (*m*, NH(Ser-Z), 5 arom. H(Z)+10 arom. H(^{t}BDPSi)); 7.86 (*s*, H–C(6)); 8.09 (br. *s*, NH$_\beta$(Asp)); 8.28 (br. *s*, NH$_\alpha$(Asp)); 8.36 (br. *s*, NH(Gly)). ^{13}C-NMR (DMSO, 75 MHz): 18.66 (Me$_3$*C*); 19.24 (Me–C(2)); 24.96 (*Me*$_2$C); 26.92 (*Me*$_3$C); 36.88 (C$_\beta$(Asp)); 37.46 (*C*H$_2$–C(5)); 41.16 (C$_\alpha$(Gly)); 51.58 (C$_\alpha$(Asp)); 52.10 (MeO); 56.45 (C$_\alpha$(Ser)); 58.33 (C(4′)); 64.29 (C$_\beta$(Ser)); 66.10 (CH$_2$(Z)); 99.87 (Me$_2$*C*); 125.75 (C(5)); 128.28–135.58 (12 arom. C(^{t}BDPSi)); 128.77–137.33 (6 arom. C(Z)); 128.77 (C(4)); 139.91 (C(6)); 145.48 (C(2)); 145.70 (C(3));

156.64 (CO(Z)); 169.67 (CO(Gly)); 170.37 (CO_α(Asp)); 171.43 (CO(Ser)); 172.45 (CO_β(Asp)). HR-MS: 854.3797 ($[M+H]^+$, $C_{45}H_{56}N_5O_{10}Si^+$; calc. 854.3796).

*pGlu-Ser(O^{t}BDPSi)-Asp[(3,4′-*O*-isopropylidene-2-methylpyridin-5-yl)methyl]-Gly-OMe* (**17**). Pd/C (10%; 180 mg, 10% by weight) was added to a stirred soln. of **16** (1.95 g, 2.28 mmol) in a degased mixture MeOH/AcOEt 1:1 (45 ml) under N_2 at r.t. The flask was purged several times with H_2, and an atmosphere of H_2 was maintained for 1 h (TLC: CH_2Cl_2/MeOH 9:1). The catalyst was filtered off over *Celite*, washed several times with CH_2Cl_2/MeOH 1:1, and the solvents were evaporated to dryness to afford the free amine as a foam (1.64 g). The latter was dissolved in DMF (23 ml), and pGlu-OH (357 mg, 2.76 mmol) was added, followed by BOP (1.2 g, 2.76 mmol). The pH of the mixture was adjusted by addition of Et_3N to 8–9. When no starting material was detected by TLC, the solvent was evaporated *in vacuo*, and the crude material was dissolved in AcOEt (100 ml). The org. layer was washed with $KHSO_4$ soln. (1N), H_2O, sat. Na_2CO_3 soln., and brine, then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was absorbed on silica and purified by silica-gel CC with CH_2Cl_2/MeOH 95:5 to afford **17** (1.5 g, 79%). White solid. ^{1}H-NMR (DMSO, 300 MHz): 0.95 (*s*, *t*-Bu); 1.47 (*s*, *Me*$_2$C); 1.89–2.03 (*m*, C*H*H$_{2\beta}$(pGlu)); 2.04–2.16 (*m*, CH$_{2\gamma}$(pGlu)); 2.21–2.34 (*m*, CH*H*$_{2\beta}$(pGlu)); 2.25 (*s*, Me–C(2)); 2.38–2.52 (*m*, CH$_{2\beta}$(Asp)); 3.57 (*s*, MeO); 3.68–3.93 (*m*, CH$_{2\alpha}$(Gly), CH$_{2\beta}$(Ser)); 4.04–4.22 (*m*, CH$_2$–C(5)); 4.12 (*m*, CH$_\alpha$(pGlu)); 4.49–4.62 (*m*, CH$_\alpha$(Asp)); 4.64–4.76 (*m*, CH$_\alpha$(Ser)); 4.82 (br. *s*, CH$_2$(4′)); 7.37–7.67 (*m*, 10 arom. H(^{t}BDPSi)); 7.84 (*s*, H–C(6)); 7.87 (br. *s*, NH$_\beta$(Asp)); 8.09 (br. *s*, NH(pGlu)); 8.28 (br. *s*, NH$_\alpha$(Asp)); 8.36 (br. *s*, NH(Ser), NH(Gly)). ^{13}C-NMR (DMSO, 75 MHz): 18.65 (Me$_3$*C*); 19.26 (Me–C(2)); 24.97 (*Me*$_2$C); 25.72 (C$_\beta$(pGlu)); 26.97 (*Me*$_3$C); 29.60 (C$_\gamma$(pGlu)); 37.23 (C$_\beta$(Asp)); 37.47 (*C*H$_2$–C(5)); 41.18 (C$_\alpha$(Gly)); 49.93 (C$_\alpha$(Asp)); 52.10 (MeO); 55.07 (C$_\alpha$(Ser)); 56.17 (C$_\alpha$(pGlu)); 58.33 (C(4′)); 64.04 (C$_\beta$(Ser)); 99.89 (Me$_2$*C*); 125.77 (C(5)); 128.32–135.54 (12 arom. C(^{t}BDPSi)); 128.32 (C(4)); 139.92 (C(6)); 145.50 (C(2)); 145.71 (C(3)); 169.53 (CO(Gly)); 169.69 (CO$_\alpha$(Asp)); 170.31 (CO(Ser)); 171.38 (CO$_\beta$(Asp)); 173.25 (CO$_\delta$(pGlu)); 177.88 (CO(pGlu)). HR-MS: 831.3740 ($[M+H]^+$, $C_{42}H_{55}N_6O_{10}Si^+$; calc. 831.3749).

*pGlu-Ser-Asp[(3,4′-*O*-diacetyl-2-methylpyridin-5-yl)methyl]-Gly-OMe* (**18**). To a soln. of **17** (3.1 g, 3.7 mmol) in abs. EtOH (37 ml) was added pyridinium 4-toluenesulfonate (2.3 g, 9.3 mmol), and the mixture was refluxed for 3 d. After cooling, the soln. was neutralized by addition of Na_2CO_3, and the solvent was evaporated under reduced pressure. The white solid was partitioned between AcOEt and H_2O, and the org. layer was decanted and washed with brine. It was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by silica-gel CC with CH_2Cl_2/MeOH 92:8 to afford the deprotected compound (1.75 g, 60%) as a white solid.

Ac_2O (1.05 ml, 11.1 mmol) was added to a stirred soln. of the deprotected compound (1.75 g, 1.05 mmol) in dry pyridine (11 ml) at r.t. under N_2. After 14 h, the pyridine was removed under reduced pressure, and the residue was dissolved in AcOEt, and the org. layer was washed with a 5% soln. of Na_2CO_3 and H_2O, and dried (Na_2SO_4). After filtration, the solvent was evaporated to dryness, and the residue was co-evaporated with toluene and MeOH. The crude white solid was used in the next reaction without further purification.

The white crude material was dissolved in THF (20 ml) at 0°, and a soln. of TBAF (1M in THF; 1.1 ml, 1.1 mmol) was added dropwise. Then, the mixture was stirred at r.t. for 20 min. The solvent was evaporated under reduced pressure, and the residue was subjected to silica-gel CC using a stepwise gradient of MeOH (10–14%) in CH_2Cl_2 to afford **18**, which was recrystallized from MeOH (0.362 g, 57%). ^{1}H-NMR (DMSO, 300 MHz): 1.89–2.03 (*m*, C*H*H$_{2\beta}$(pGlu)); 2.04–2.16 (*m*, CH$_{2\gamma}$(pGlu)); 1.97 (*s*, MeCO); 2.21–2.34 (*m*, CH*H*$_{2\beta}$(pGlu)); 2.28 (*s*, Me–C(2)); 2.36 (*s*, MeCO); 2.38–2.52 (*m*, CH$_{2\beta}$(Asp)); 3.32 (*s*, MeO); 3.58–3.91 (*m*, CH$_{2\alpha}$(Gly), CH$_{2\beta}$(Ser)); 4.02–4.16 (*m*, CH$_2$–C(5)); 4.10–4.15 (*m*, CH$_\alpha$(pGlu)); 4.49–4.62 (*m*, CH$_\alpha$(Asp)); 4.63–4.68 (*m*, CH$_\alpha$(Ser)); 5.05 (br. *s*, OH(Ser)); 5.12 (br. *s*, CH$_2$(4′)); 7.79 (*s*, H–C(6)); 8.01 (br. *s*, NH$_\beta$(Asp)); 8.25 (br. *s*, NH(pGlu)); 8.29 (br. *s*, NH$_\alpha$(Asp), NH(Gly)); 8.41 (br. *s*, NH(Ser)). ^{13}C-NMR (DMSO, 75 MHz): 19.51 (Me–C(2)); 20.76 (*Me*CO); 20.78 (*Me*CO); 25.62 (C$_\beta$(pGlu)); 29.63 (C$_\gamma$(pGlu)); 37.36 (C$_\beta$(Asp)); 37.78 (*C*H$_2$–C(5)); 40.80 (C$_\alpha$(Gly)); 49.99 (C$_\alpha$(Asp)); 52.15 (MeO); 55.42 (C$_\alpha$(Ser)); 55.89 (C$_\alpha$(pGlu)); 57.07 (C(4′)); 60.89 (C$_\beta$(Ser)); 132.89 (C(5)); 135.05 (C(4)); 144.47 (C(6)); 147.18 (C(2)); 151.07 (C(3)); 169.15 (2 Me*C*O); 169.77 (CO(Gly)); 170.20 (CO$_\alpha$(Asp)); 170.34 (CO(Ser)); 171.68 (CO$_\beta$(Asp)); 173.23 (CO$_\delta$(pGlu)); 177.98 (CO(pGlu)). HR-MS: 637.2456 ($[M+H]^+$, $C_{27}H_{36}N_6O_{12}^+$; calc. 637.2469).

*pGlu-Ser[*O*-(2-cyanoethyl)thymidinephosphotriester]-Asp[(3,4′-*O*-diacetyl-2-methylpyridin-5-yl)-methyl]-Gly-OMe* (**19**). 2-Cyanoethyl diisopropylchlorophosphoramidite (0.172 ml, 0.77 mmol) was added to a stirred soln. of Et_3N (0.124 ml, 0.88 mmol) and 3′-*O*-acetylthymidine (0.188 g, 0.66 mmol) in dry MeCN (2.5 ml) at 0° under N_2 (→ thymidine-5′-phosphoramidite (**9**)). The mixture was stirred at r.t. for 20 min and was cooled in an ice bath. Then, a 0.45M soln. of 1*H*-tetrazole in dry MeCN (5 ml) was added, followed by the addition of **18** (0.350 g, 0.55 mmol) in dry DMF (4 ml). The mixture was stirred at r.t. for 2 h, and a 0.02M oxidative standard soln. (I_2/H_2O/pyridine, 11 ml) was added. After 30 min stirring, the mixture was diluted with CH_2Cl_2 (22 ml), and the org. layer was washed with a 20% bisulfite soln. and brine, and dried (Na_2SO_4) and evaporated to dryness. The residue was subjected to silica-gel CC with CH_2Cl_2/MeOH 9:1 to afford **19** (0.130 g, 23%). White solid. ^{1}H-NMR (DMSO, 300 MHz): Gly: 3.38 (*s*, MeO); 3.65–3.88 (*m*, $CH_{2\alpha}$); 8.34 (overl., NH); pyridine moiety: 1.97 (*s*, MeCO); 2.31 (*s*, Me); 2.36 (*s*, MeCO); 4.04–4.18 (*m*, CH_2–C(5)); 5.05 (*s*, C*H*$_2$OAc); 7.52 (*s*, H–C(6)); Asp: 2.38–2.52 (*m*, $CH_{2\beta}$); 4.49–4.62 (*m*, CH_α); 8.13 (br. *s*, NH_β); 8.34 (br. *s*, NH_α); Ser: 3.43–3.51 (*m*, $CH_{2\beta}$); 4.68 (br. *s*, CH_α); 8.42 (*s*, NH); pGlu: 2.04, 2.12 (*m*, $CH_{2\gamma}$); 2.21–2.34 (*m*, $CH_{2\beta}$); 4.38–4.43 (*m*, CH_α); 8.29 (*s*, NH); Thy: 1.78 (*s*, Me); 2.04, 2.12 (*m*, CH_2(2′)); 2.07 (*s*, MeCO); 2.90 (br. *s*, CH_2CN); 4.04–4.10 (*m*, CH_2(5′)); 4.15–4.20 (*m*, CH(4′)); 4.22 (br. *s*, CH_2OP); 5.19 (br. *s*, H–C(3′)); 6.20 (*t*, *J* = 7, H–C(1′)); 7.73 (*s*, H–C(6)); 11.36 (*s*, NH). ^{13}C-NMR (DMSO, 75 MHz): Gly: 41.05 (C_α); 52.13 (MeO); 169.64 (CO); pyridine moiety: 19.51 (Me); 20.76 (*Me*CO); 20.78 (*Me*CO); 37.52 (CH_2–C(5)); 57.07 (C(4′)); 131.31 (C(5)); 136.00 (C(4)); 144.69 (C(6)); 147.36 (C(2)); 150.87 (C(3)); 169.15 (Me*C*O); 170.81 (Me*C*O); Asp: 37.36 (C_β); 49.99 (C_α); 170.20 (CO_α); 171.68 (CO_β); Ser: 52.87 (C_α); 67.94 (C_β); 168.60 (CO); pGlu: 25.55 (C_β); 29.56 (C_γ); 63.04 (C_α); 173.25 (CO_δ); 177.87 (CO); Thy: 12.50 (Me); 19.51 (*C*H_2CN); 21.20 (*Me*CO); 36.04 (C(2′)); 67.51 (CH_2OP); 69.67 (C(5′)); 84.43 (C(3′)); 83.86 (C(1′)); 85.9 (C(4′)); 110.61 (C(5)); 118.62 (*C*N); 135.92 (C(6)); 152.36 (C(2)); 164.08 (C(4)); 169.14 (Me*C*O). HR-MS: 1036.3302 ($[M+H]^+$, $C_{42}H_{54}N_9O_{20}P^+$; calc. 1036.3301).

*pGlu-Ser(*O*-thymidinephosphodiester)-Asp[(3-hydroxy-4-(hydroxymethyl)-2-methylpyridin-5-yl)-methyl]-Gly-OH* (**20**). Compound **19** (0.050 g, 0.048 mmol) was dissolved in MeOH (2.4 ml), and the mixture was cooled to 0°. Then, an aq. soln. of K_2CO_3 10% (2.4 ml) was added dropwise, and the mixture was stirred overnight (the temp. was raised slowly to r.t.). The pH was then adjusted to 3 with *DOWEX* H^+ form, and the solvent was removed. The residue was purified on silica gel with $CHCl_3$/MeOH/H_2O 5:4:1 to afford **20**, which was lyophilized from H_2O (0.011 g, 29%). HR-MS: 841.2412 ($[M-H]^-$, $C_{32}H_{43}N_8O_{17}P^-$; calc. 841.2405).

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