Peptide–Oligonucleotide Hybrids with N-Acylphosphoramidate Linkages

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The preparation of a peptide–oligonucleotide hybrid with an N-acylphosphoramidate linkage has been carried out by (i) phosphitylation of the C-terminal carboxamide of a protected peptide by reaction with a chloroalkoxy(dialkylamino)phosphine in the presence of a base, (ii) coupling of the resulting N-acylphosphorodiamidite onto an oligonucleotide-resin followed by oxidation, and (iii) deprotection and cleavage under basic conditions. The synthetic method takes advantage of the unprecedented reaction of primary carboxamides with electrophilic P^{III} species and takes place under mild conditions which are compatible with the stability of both the peptide and the oligonucleotide components.

Among the few nucleotide antibiotics described, agrocin-84 (**1**)[1] and phosmidosine (**2**)[2] share the presence of an N-acylphosphoramidate function (Figure 1). In the particular case of phosmidosine, this functional group links a nucleoside analogue and an amino acid residue. To our knowledge, no synthesis of peptide–oligonucleotide hybrids with N-acylphosphoramidate linkages has been described yet, so their chemical or biological properties remain to be evaluated.

Syntheses of N-acylphosphoramidate derivatives have been described, as outlined below (Figure 2), by either acylation of a phosphoramidate group,[3,4] reaction of carboxamide salts with phosphorochloridates,[5,6] esterification of N-acylphosphoramidic acids (or derivatives),[7] or reaction of trialkyl phosphites with N-haloamides.[8]

The acylation of neutral phosphoramidates (eq 1) has been studied under different conditions and has been found to be accompanied by cleavage of the P–N bond and formation of a carboxamide.[3] The reaction of acyl halides with the conjugate base of phosphoramidates gives a mixture of products,[4] and consequently, it is also not practical for preparative purposes.

Phosphorochloridate esters and the conjugate base of primary or secondary carboxamides[5,6] react to give the desired N-acylphosphoramidate (eq 2). This synthetic method requires the use of strong bases (sodium, butyllithium) for the preparation of the amide anion and thus is only compatible with base-stable substrates.

N-Acylphosphoramidic acids or chloroacylphosphoramidates (ClP(O)(OH)NHCOR″) can be esterified with an alcohol to N-acylphosphoramidates[7] (eq 3). The inconvenience of the method lies in the preparation of the dichloroacylphosphoramidate precursors (or their corresponding monochloro or acid derivatives), which can only be obtained from aromatic carboxamides or aliphatic amides with electron-withdrawing substituents at the α-carbon. With other substrates, a large or nearly quantitative amount of dehydration takes place.[9]

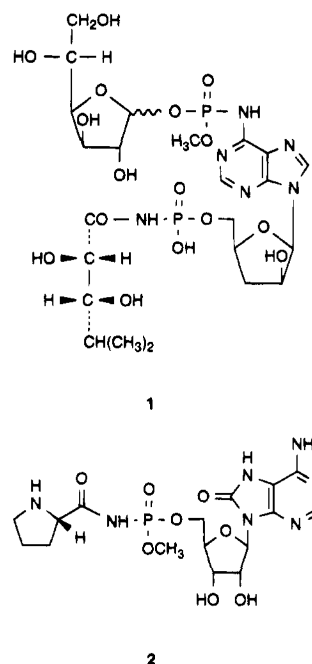

Figure 1. Structures of the nucleotide antibiotics agrocin-84 (**1**) and phosmidosine (**2**).

With respect to the Arbuzov reaction between N-haloamides and trialkyl phosphites (eq 4), N-acylphosphoramidates can only be obtained from cyclic haloamides or cyclic haloimides.[8]

As a result, none of these methods is of general application nor seems to be adequate for the preparation of peptide–oligonucleotide hybrids linked through an N-acylphosphoramidate function, either because they are only compatible with certain substrates or because the corresponding method is accompanied to a large extent

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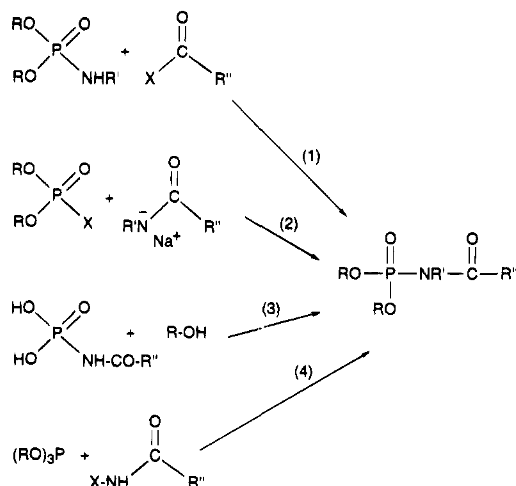

Figure 2. Summary of the methods used for the preparation of *N*-acylphosphoramidates.

by side reactions. We will report in this paper on new alternatives, suitable for the preparation of *N*-acylphosphoramidate peptide−oligonucleotide hybrids, which are based on the reaction of primary carboxamides with electrophilic P^{III} species.

One of our research interests is to develop a simple and general method for the synthesis of nucleopeptides (peptide−oligonucleotide hybrids linked through a phosphodiester bond between the side chain hydroxyl group of an amino acid residue and a terminal hydroxyl group of an oligonucleotide chain). For this purpose, we have evaluated two different synthetic schemes, a convergent and a stepwise approach, having in common the extended use of solid supports and the protection scheme.[10−13]

To carry out the synthesis of a nucleopeptide by a convergent approach, it is necessary to obtain a protected peptide with a free side chain hydroxyl group in order to allow its phosphitylation and coupling to a conveniently protected oligonucleotide. When we carried out the reaction of peptide Ac-Ser-Gly-Asp(OFm)-NH_2[14,15] with chloro(2-cyanoethoxy)diisopropylaminophosphine and a tertiary base and analyzed the crude reaction mixture by ^{31}P-NMR, we found the 145−148 ppm signal corresponding to the desired O-phosphitylated derivative plus another at 116−117 ppm which was assigned to an impurity originating in the reaction of the carbamoyl group ($CONH_2$) with the chlorophosphine. van Boom and co-workers[16] have described the presence of an unknown impurity having the same chemical shift when they phosphitylated a peptide-amide under similar reaction conditions. We have observed that when (2-cyanoethoxy)bis(diisopropylamino)phosphine and tetrazole are used to obtain the peptide-phosphoramidite, the phosphitylation of the carboxamide takes place much more slowly.

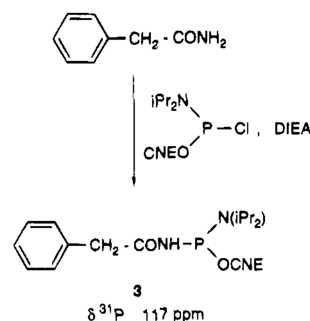

Figure 3. Phosphitylation of carbamoyl groups by chloroalkoxy(dialkylamino)phosphines.

We first studied the reaction between chlorophosphines and carbamoyl groups on phenylacetamide, a product structurally simpler and more easily accessible than a protected peptide. We found that phenylacetamide does effectively react with chloro(2-cyanoethoxy)diisopropylaminophosphine and ethyldiisopropylamine to give a product (**3**) with a phosphorus chemical shift of about 117 ppm (Figure 3). The comparison with other chemical shifts[17] suggests a structure of *N*-acylphosphorodiamidite in which the nitrogen (and not the oxygen) atom is phosphitylated, and the presence at the ^{13}C-NMR spectrum of a signal with the chemical shift of a carbonyl group confirms this assignment. It is not necessarily surprising to get N- instead of O-phosphitylation since acylation and alkylation of primary or secondary carboxamides in the presence of a base are known to take place at the nitrogen atom.[18]

The synthesis of oligonucleotides is commonly accomplished on a solid support by the phosphite triester methodology,[19] in which dialkoxy(dialkylamino)phosphines (phosphoramidites) react with alcohols in the presence of an acid catalyst such as tetrazole to give the phosphite triester. Thus, we have explored whether, in a similar way, *N*-acylphosphorodiamidites such as **3** would react with alcohols. The reaction of $PhCH_2$-CONHP(OCNE)$NiPr_2$ (**3**) and the secondary 3′-hydroxyl group of 5′-*O*-(dimethoxytrityl)thymidine, in the presence of tetrazole, afforded an intermediate (^{31}P-NMR δ 130.1, 131.9 ppm) which, after oxidation with *tert*-butyl hydroperoxide, was transformed into the *N*-acylphosphoramidate diester **4** (^{31}P-NMR δ −2.5, −3.2 ppm) (Figure 4).

We have already mentioned that carbamoyl groups can be phosphitylated, although more slowly, with alkoxybis(dialkylamino)phosphines and tetrazole. Consequently, it also seemed reasonable that phosphoramidite derivatives, such as those commonly used to perform the synthesis of oligonucleotides, would be able to react with primary carboxamides. We carried out the reaction between phenylacetamide, tetrazole, and 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite), and after oxidation of the intermediate, we

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(14) Abbreviations: ACN, acetonitrile; CNE, 2-cyanoethyl; DCM, dichloromethane; Dmf, (*N*,*N*-dimethylamino)methylene; DMT, 4,4′-dimethoxytrityl; DIEA, *N*-ethyl-*N*,*N*-diisopropylamine; Fm, 9-fluorenylmethyl; iBu, isobutyryl; P, polystyrene-*co*-1%-divinylbenzene; suc, succinyl; TCA, trichloroacetic acid.
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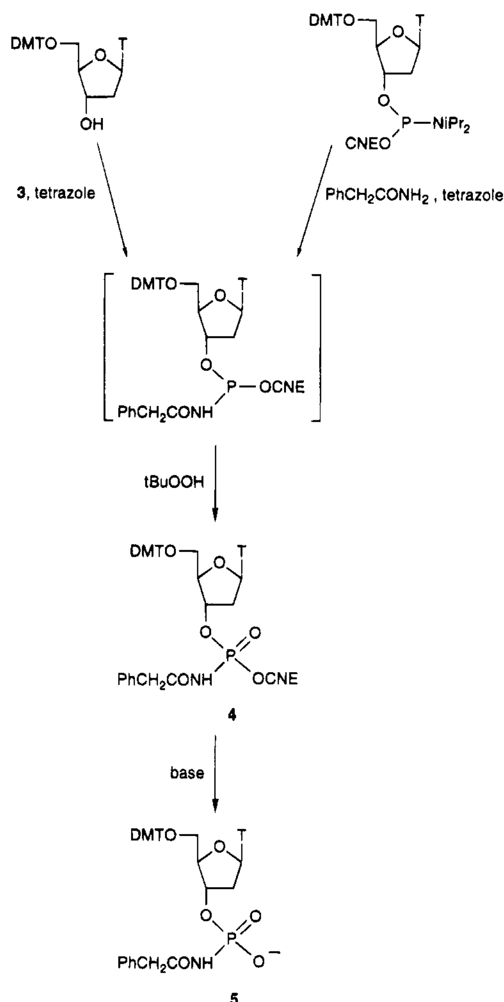

Figure 4. Synthesis of the *N*-acylphosphoramidate diester **4** by reaction of either phosphitylated phenylacetamide (**3**) and an alcohol or phenylacetamide and a phosphoramidite derivative in the presence of tetrazole, followed by oxidation. Treatment with base (see text) yields the stable *N*-acylphosphoramidate monoester, **5**.

again obtained the *N*-acylphosphoramidate diester **4** (Figure 4).

With respect to the stability of *N*-acylphosphoramidates, treatment of **4** with a basic reagent removed the cyanoethyl group to afford the *N*-acylphosphoramidate monoester **5** (Figure 4). This derivative was found to be stable to different basic conditions (1:1 concentrated aqueous ammonia/dioxane at 55 °C, 15 h; 0.25 M LiOH in 1.5:1.5:1 methanol/dioxane/water, 24 h; 0.05 M K_2CO_3 in 1:1 methanol/dioxane at room temperature, 24 h). A treatment with 80% aqueous acetic acid for 15 h at room temperature eliminated the DMT group but left unaltered the acylphosphoramidate monoester group.

These results, and the successful synthesis of a peptide-nucleoside conjugate,[13] prompted us to undertake the synthesis of a more complex molecule, a peptide–oligodeoxynucleotide hybrid with the two components linked through an *N*-acylphosphoramidate function. We chose the synthetic scheme in which the key step was to couple a fully protected peptide with a phosphitylated C-terminal carbamoyl group onto an oligonucleotide-resin with all the functional groups protected except the 5′-terminal hydroxyl group (see Figure 5). This synthetic scheme offers the advantage that both the protected peptide and the oligonucleotide-resin are easily accessible (there is a relatively large experience in the synthesis of protected peptides[20,21]) and that the use of the solid-phase methodology simplifies the overall procedure, allowing the easy elimination of the excess of phosphitylated peptide. All the permanent protecting groups were chosen to be removed under basic conditions which have been shown not to degrade the target molecule. The side chain hydroxyl and carboxyl groups of serine and aspartic acid were protected as acetate and as 9-fluorenylmethyl ester,[22,23] respectively; (dimethylamino)methylene and isobutyryl groups[24] were used for the protection of the nucleobases; a succinyl group linked the 3′-terminal hydroxyl group to a polystyrene support, and all the phosphate functions were protected as 2-cyanoethyl esters.

Once the side chain hydroxyl group of peptide Ac-Ser-Gly-Asp(OFm)-NH_2[15] was protected as an acetate (**6**), the peptide-phosphorodiamidite (**7**) was obtained by reaction with chloro(2-cyanoethoxy)diisopropylaminophosphine and *N*-ethyl-*N*,*N*-diisopropylamine. In this process, we have not detected any reaction between the chlorophosphine and the secondary carboxamide groups of the peptide chain. The tetrazole-mediated reaction of **7** with the oligodeoxynucleotide-resin (**8**) was carried out in an automatic synthesizer. After the capping and oxidation steps, the product was deprotected by treatment with 1:1 concentrated aqueous ammonia/dioxane at 55 °C and purified by medium pressure reversed-phase liquid chromatography. The peptide–oligonucleotide hybrid Ac-Ser-Gly-Asp-NH-$p^{5'}$CATCAT (**9**) had the correct amino acid and nucleoside composition (after acid and enzymatic hydrolysis, respectively), and the electrospray mass spectrum showed a series of peaks from which the estimated mass was in accord with the expected theoretical mass. It was confirmed that peptide–oligonucleotide conjugate **9** has the same stability to bases as the *N*-acylphosphoramidate monoester derivative **5**. Upon treatment with 80% acetic acid, 33% of **9** was degraded after 22 h at room temperature, while at 55 °C, there was 28% degradation after 1 h and complete degradation after 15 h. In this respect, peptide–oligonucleotide **9** is more susceptible to acid degradation than the model compound **5**, but it is noticeable that compounds with *N*-acylphosphoramidate groups seem to be more stable to acids than those containing phosphoramidate functional groups: oligonucleotides with phosphoramidate bonds have been reported to be quantitatively cleaved by 80% acetic acid at room temperature in 5–6 h.[25–27]

In summary, we have shown that *N*-acylphosphoramidate diesters can be obtained by either (i) phosphitylation of carbamoyl groups with chloroalkoxy(dialkylamino)phosphines and a tertiary base and reaction of the resulting phosphorodiamidite with an alcohol and tetrazole followed by oxidation, or (ii) tetrazole-mediated reaction of a phosphoramidite and a primary carboxamide and subsequent oxidation. Using the first method,

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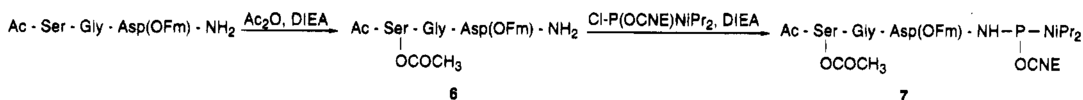
i) Synthesis of the protected peptide-phosphorodiamidite 7



ii) Coupling of the peptide-phosphorodiamidite onto the oligonucleotide-resin and deprotection

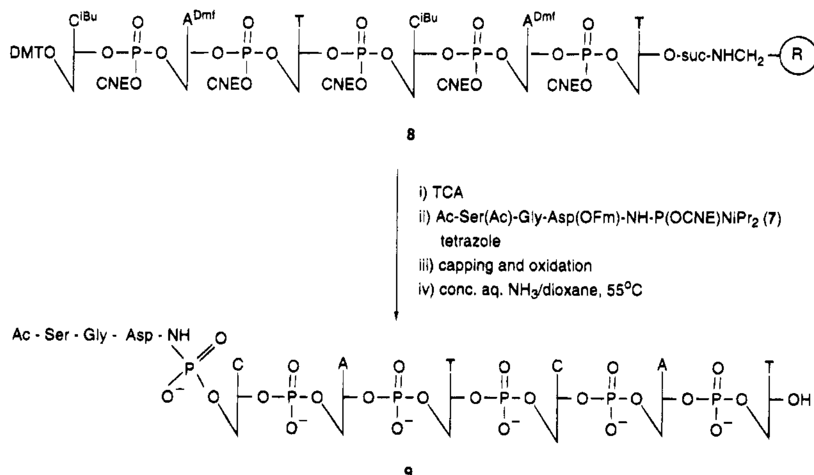


Figure 5. Synthesis of the nucleopeptide analogue Ac-Ser-Gly-Asp-NH-p$^{5'}$CATCAT, **9**.

we have synthesized, for the first time, a peptide–oligonucleotide hybrid with an *N*-acylphosphoramidate union. The target peptide–oligonucleotide hybrid has been shown to be stable under a variety of basic conditions and labile to treatment with an acidic reagent, but the presence of the nitrogen-linked acyl group increases the acid stability with respect to unmodified phosphoramidate groups. Work is in progress to extend the applicability of the method to the widest range of molecules, in particular, peptide–oligonucleotide hybrids whose biological or therapeutic applications have, to date, not been explored.

Experimental Section

General Information. Aminomethylpolystyrene-*co*-1%-divinylbenzene was obtained from Novabiochem AG. 5′-*O*-Dimethoxytrityl 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) derivatives of 6-*N*-[(*N,N*-dimethylamino)methylene]-2′-deoxyadenosine (dA^{Dmf}), 4-*N*-isobutyryl-2′-deoxycytidine (dC^{iBu}), and thymidine were purchased from Applied Biosystems and Cruachem. Solid-phase oligonucleotide synthesis and coupling of protected peptide-acylphosphorodiamidite onto oligonucleotide-resin were performed on a 380B Applied Biosystems synthesizer following standard procedures with modifications rendering the phosphite triester methodology compatible with the use of polystyrene.[28,29] Dry acetonitrile was obtained by distillation and storage over CaH_2. Dichloromethane was neutralized and dried by passing it through basic alumina and storing it over CaH_2. Dry tetrahydrofuran was obtained directly by distillation over Na metal in the presence of benzophenone. Dimethylformamide was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4 Å molecular sieves. All solvents were filtered prior to use in the automatic synthesizer. Reversed-phase HPLC analyses were performed with C_{18} columns using linear gradients of 0.045% trifluoroacetic acid in water and 0.036% trifluoroacetic acid in acetonitrile for peptide Ac-Ser(Ac)-Gly-Asp(OFm)-NH_2 (system RPA, detection wavelength 220 and 300 nm) and 0.01 M aqueous triethylammonium acetate and acetonitrile (system RPB) or 1:1 acetonitrile/H_2O (system RPC) for the other derivatives (detection wavelength 260 nm). Amino acid analysis of the nucleopeptide analogue was performed after hydrolysis in 12 M 1:1 HCl/propionic acid at 150 °C for 90 min.

(2-Cyanoethoxy)(diisopropylamino)(phenylacetamido)-phosphine (3). Phenylacetamide (300 mg, 2.2 mmol) was dried by coevaporation with anhydrous acetonitrile and dissolved in anhydrous DCM. Then, under an argon atmosphere, 1 mL of (2-cyanoethoxy)chloro(diisopropylamino)phosphine (4.4 mmol) and 1.1 mL of DIEA (6.5 mmol) were added. After stirring for 1 h at room temperature, the reaction mixture was diluted with more DCM, and the excess of phosphine was eliminated by reaction with (hydroxymethyl)polystyrene (5 equiv) for an additional 1 h. The resin was removed by filtration, and the resulting solution was washed with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to dryness to give a yellowish oil (85% yield). The product could be purified (90–95% by ^{13}C-NMR) by fast column chromatography through activated neutral alumina, eluting with 100:2 hexanes/pyridine and a mixture of 50:50:2 hexanes/AcOEt/pyridine. Elimination of the solvent and coevaporation of the residue with acetonitrile gave a yellowish oil (53% yield): R_f (45:45:10 DCM/AcOEt/Et_3N, the spot was visualized using iodine) 0.75; ^{31}P-NMR (121.4 MHz, $CDCl_3$) δ 117.3; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.30 (m, 5H), 6.05 (br s, 1H), 3.77 (t, 2H, J = 6.3 Hz), 3.50 (s, 2H), 3.34 (m, 2H), 2.50 (t, 2H, J = 6.3 Hz), 1.09 (d, 6H, J = 6.6 Hz), 0.97 (d, 6H, J = 6.6 Hz); ^{13}C-NMR (75.4 MHz, $CDCl_3$) δ 173.3 (d, $^2J_{CP}$ = 11.9 Hz), 134.6, 128.9, 128.5, 126.9, 117.4, 59.6 (d, $^2J_{CP}$ = 26.8 Hz), 44.2, 43.9 (d, $^2J_{CP}$ = 13.1 Hz), 23.9 (d, $^3J_{CP}$ = 7.6 Hz), 23.7 (d, $^3J_{CP}$ = 6.7 Hz), 19.9 (d, $^3J_{CP}$ = 7.9 Hz).

2-Cyanoethyl 5′-*O*-(Dimethoxytrityl)thymidin-3′-yl *N*-(Phenylacetyl)phosphoramidate (4). (a) From 5′-*O*-(Dimethoxytrityl)thymidine and 3. (2-Cyanoethoxy)-(diisopropylamino)(phenylacetamido)phosphine (**3**, 1 mmol) and 5′-*O*-(dimethoxytrityl)thymidine (273 mg, 0.5 mmol) were dried by coevaporation with anhydrous ACN and dissolved in anhydrous DCM. Tetrazole (280 mg, 4 mmol) was added, and the mixture was stirred for 1 h at room temperature under an argon atmosphere. tBuOOH (3 M solution in toluene, 10 equiv) was added and, after 15 min, the reaction mixture was

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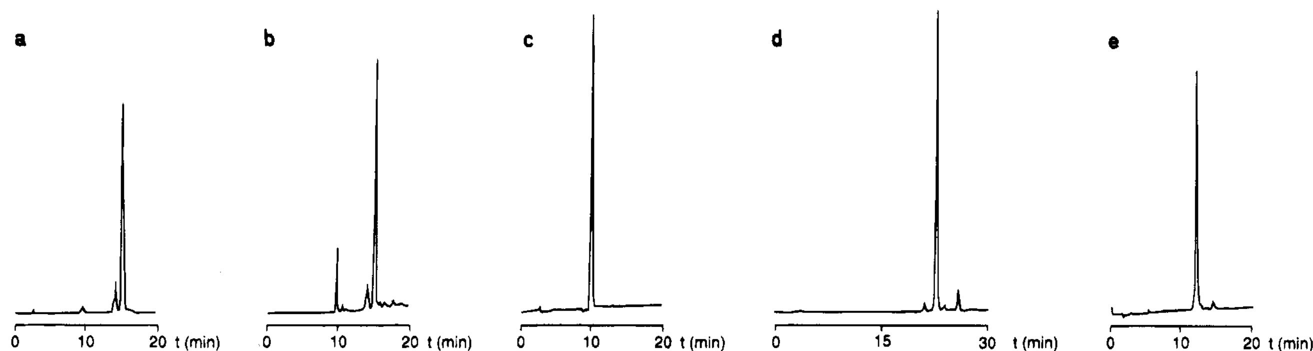

Figure 6. Analytical HPLC profiles (analysis conditions and t_R for each product are indicated in the text): (a) *N*-acylphosphoramidate diester **4** obtained from route a (see text), (b) *N*-acylphosphoramidate diester **4** from route b, c) *N*-acylphosphoramidate monoester **5** (lithium salt), (d) Ac-Ser(Ac)-Gly-Asp(OFm)-NH_2, **6**, and (e) Ac-Ser-Gly-Asp-NH-p[5']CATCAT, **9**.

diluted with dichloromethane, washed with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in dichloromethane and precipitated over hexanes to give a white solid (80% yield). No further purification was attempted since the product was shown to be fairly unstable.

(b) From Phenylacetamide and 5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-(2-Cyanoethyl *N,N*-diisopropylphosphoramidite). 5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (150 mg, 0.2 mmol) and phenylacetamide (55 mg, 0.4 mmol) were dried by coevaporation with anhydrous ACN and dissolved in anhydrous DCM. Tetrazole (56 mg, 0.8 mmol) was added, and the mixture was stirred for 1 h at room temperature under an argon atmosphere. The same oxidation and workup procedure as above was followed to obtain **4** (85% yield).

4: R_f (9:1 DCM/MeOH) 0.45; R_f (9:1 AcOEt/hexane) 0.15; mp 86−88 °C; ^{31}P-NMR ($CDCl_3$, 81 MHz) δ −2.5, −3.2; ^{1}H-NMR (200 MHz, $CDCl_3$) δ 9.25 (s, 1H), 7.56 (s, 1H), 7.30−7.20 (m, 14H), 6.83 (d, 4H, J = 8 Hz), 6.45 (m, 1H), 4.45−4.10 (m, 4H), 3.77 (s, 6H), 3.50−3.20 (m, 4H), 2.65−2.30 (m, 4H), 1.40 (s, 3H); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 173.0, 164.0, 158.8, 150.5, 144.1, 135.5, 135.0, 130.1, 129.3, 128.9, 128.1, 128.0, 127.6, 127.3, 116.5, 113.3, 111.6, 87.2, 84.4, 79.5, 77.2, 63.3, 62.5, 55.3, 43.9, 39.2, 19.5, 11.7; IR (film) 2320 (CN st), 1265 (P=O st) cm^{-1}; analytical HPLC (system RPB, linear gradient from 20 to 100% B in 20 min) t_R 14.9 min (Figure 6, chromatograms a and b).

5′-*O*-(Dimethoxytrityl)thymidin-3′-yl *N*-(Phenylacetyl)-phosphoramidate (5). A solution of **4** (100 mg, 0.13 mmol) in 10 mL of 1:1 concentrated aqueous NH_3/dioxane was stirred for 15 h at room temperature, after which time the solvent was evaporated. The residue was dissolved in DCM, washed with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to dryness. The resulting residue was dissolved in DCM and precipitated over hexanes to give a white solid (ammonium salt, 85% yield). *N*-Acylphosphoramidate monoester **5** (lithium salt) was also obtained, with a high degree of purity, from *N*-acylphosphoramidate diester **4** after a treatment with 0.25 M LiOH in MeOH/dioxane/H_2O for 4 h at room temperature.

5: mp 94−96 °C; ^{31}P-NMR (81 MHz, CD_3OD) δ −8.8; ^{1}H-NMR (200 MHz, CD_3OD) δ 9.65 (s, 1H), 7.4−7.0 (m, 14H), 6.85 (d, 4H, d, J = 8 Hz), 6.35 (m, 1H), 3.82 (s, 6H), 3.50 (m, 2H), 3.40−3.20 (m, 2H), 2.40−2.20 (m, 2H), 1.42 (s, 3H); ^{13}C-NMR (50 MHz, CD_3OD) δ 175.3, 166.6, 160.2, 152.5, 145.9, 137.6, 136.7, 136.6, 136.3, 131.4, 130.3, 129.4, 128.9, 127.9, 127.8, 114.2, 111.8, 88.1, 86.6, 85.8, 77.8, 64.8, 55.7, 44.2, 40.6, 11.5; FAB-MS ("magic bullet", Xe, negative mode) m/z 740 (M − H)$^-$, 614.7 (M − thymine)$^-$, 438.4 (M − DMT)$^-$; analytical HPLC (system RPB, linear gradient from 20 to 100% B in 20 min) t_R 9.9 min (elution profile c in Figure 6).

Stability of Compound 5. One-milligram aliquots of **5** (1.4 μmol) were dissolved in the corresponding reagent (25−100 equiv): (i) 0.05 M K_2CO_3 in 1:1 MeOH/dioxane, (ii) 0.25 M LiOH in 1.5:1.5:1 MeOH/dioxane/H_2O, or (iii) 1:1 concentrated aqueous NH_3/dioxane at 55 °C. The evolution of the product was followed by HPLC analysis of aliquots of the solutions (previously neutralized by addition of 1% aqueous AcOH, system RPB, linear gradient from 20 to 100% of B in 20 min). Five milligrams of **5** was treated with 500 μL of 80% aqueous acetic acid for 15 h at room temperature, after which time the solution was diluted with H_2O, washed with ether, and lyophilized to yield $PhCH_2CONHPO(O^-)T$: analytical HPLC (system RPC, isocratic 2.5% B for 10 min, linear gradient from 2.5 to 12.5% of B in 10 min, linear gradient from 12.5 to 100% of B in 5 min, and 5 min isocratic 100% B) t_R 26.1 min (t_R of thymidine under the same analysis conditions 19.2 min); electrospray MS (negative mode) m/z 899 (2M − 2H + Na)$^-$, 877 (2M − H)$^-$, 438 (M − H)$^-$.

Ac-Ser(Ac)-Gly-Asp(OFm)-NH_2 (6). Ac_2O (120 μL, 5 equiv) and DIEA (210 μL, 5 equiv) were added to a solution of peptide Ac-Ser-Gly-Asp(OFm)-NH_2[15] in DMF (120 mg, 0.25 mmol). After the mixture was stirred for 1.5 h at room temperature, MeOH was added and the mixture was evaporated to dryness. The resulting residue was treated with anhydrous ether, and peptide Ac-Ser(Ac)-Gly-Asp(OFm)-NH_2 was obtained after centrifugation as a white solid (85% yield): ^{1}H-NMR (200 MHz, DMSO-d_6) δ 8.40−8.10 (m, 3H), 7.88 (d, 2H, J = 7 Hz), 7.65 (d, 2H, J = 7 Hz), 7.43−7.10 (m, 6H), 4.53−4.00 (m, 7H), 3.70 (m, 2H), 2.87−2.52 (m, 2H), 1.98 (s, 3H), 1.85 (s, 3H); ^{13}C-NMR (50 MHz, DMSO-d_6) δ 172.4, 170.6, 170.4, 170.1, 169.6, 168.7, 143.8, 140.9, 128.0, 127.4, 125.5, 120.4, 66.2, 59.7, 52.0, 49.2, 46.3, 42.5, 36.2, 22.7, 20.9; FAB-MS (nitrobenzyl alcohol, Xe, positive mode) m/z 539.4 (M + H)$^+$, 522.4 (M − NH_2)$^+$; analytical HPLC (system RPA, linear gradient from 10 to 78% of B in 30 min) t_R: 22.2 min (see chromatogram d in Figure 6; under the same analysis conditions, t_R of peptide Ac-Ser-Gly-Asp(OFm)-NH_2 21.0 min).

Ac-Ser(Ac)-Gly-Asp(OFm)-NH-P(OCNE)NiPr$_2$ (7). Fifty milligram (93 μmol) of tripeptide Ac-Ser(Ac)-Gly-Asp(OFm)-NH_2 **6** were dried by coevaporation with anhydrous ACN and suspended in anhydrous DCM. DIEA (50 μL, 3 equiv) and (2-cyanoethoxy)chloro(diisopropylamino)phosphine (45 μL, 2 equiv) were added under an argon atmosphere, and the mixture was stirred for 1 h at room temperature, after which time the peptide was solubilized. The reaction mixture was diluted with more DCM, and (hydroxymethyl)polystyrene was added to eliminate the excess of phosphine. After the mixture was stirred for 1 h, the resin was removed by filtration, and the resulting solution was washed with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to dryness to give Ac-Ser(Ac)-Gly-Asp(OFm)-NH-P(OCNE)NiPr$_2$: ^{31}P-NMR (81 MHz, $CDCl_3$) δ 118.4, 118.0.

Ac-Ser-Gly-Asp-NH-p[5']CATCAT (9). The fully protected oligonucleotide-resin (**8**) was obtained from synthesis on a polystyrene-*co*-divinylbenzene solid support by the phosphite triester methodology,[28,29] using the 5′-*O*-(dimethoxytrityl) 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) derivatives of thymidine, 4-*N*-isobutyryl-2′-deoxycytidine and 6-*N*-[(*N,N*-dimethylamino)methylene]-2′-deoxyadenosine. After the oligonucleotide chain assembly and removal of the 5′-DMT

protecting group, 200 μL of a 0.25 M solution of Ac-Ser(Ac)-Gly-Asp(OFm)-NH-P(OCNE)$NiPr_2$ in anhydrous THF (the peptide-phosphorodiamidite was previously dried by coevaporation with anhydrous ACN) and 200 μL of a 1 M solution of tetrazole in anhydrous THF were added onto 15 mg of oligonucleotide-resin (1.5 μmol) in an automatic synthesizer. After 4 h of coupling, capping of unreacted hydroxyl groups, and oxidation, the peptide–oligonucleotide-resin was treated with 5 mL of 1:1 concentrated aqueous NH_3/dioxane for 15 h at 55 °C. The resin was separated by filtration, and the resulting solution was evaporated to dryness. The residue was dissolved in H_2O, and after filtration to remove insoluble material, the product was purified by reversed-phase medium pressure liquid chromatography: solvent A, 0.05 M triethylammonium acetate; solvent B, 1:1 ACN/H_2O; elution with a linear gradient from 15 to 25% of B. The target nucleopeptide analogue was obtained in a 26% overall yield (15 OD_{260}): amino acid composition after acid hydrolysis Ser 0.72, Asp 0.97, Gly 1.03; nucleoside composition after enzymatic digestion with snake venom phosphodiesterase and alkaline phosphatase dC 0.9, T 1.0, dA 1.1; the product remained completely stable after a treatment with calf spleen phosphodiesterase; ^{1}H-NMR (sodium salt, obtained by anion exchange chromatography through Dowex 50Wx4 and lyophilization from D_2O, 500 MHz, D_2O) δ 8.27 (s, 1H), 8.25 (s, 1H), 7.86 (s, 2H), 7.44 (d, 1H, J = 7.5 Hz), 7.38 (d, 1H, $J = J$ = 7.5 Hz), 7.34 (s, 1H), 7.18 (s, 1H), 6.20, 6.01–5.90 (m, 6H), 5.82 (d, 1H, J = 7.5 Hz), 5.78 (d, 1H, J = 7.5 Hz), 4.50–3.00 (30H), 2.72–2.09 (14H), 1.93 (s, 3H), 1.58 (s, 3H), 1.52 (s, 3H); electrospray MS (negative mode) m/z 723.9 $(M - 5H + 2Na)^{3-}$, 716.8 $(M - 4H + Na)^{3-}$, 709.1 $(M - 3H)^{3-}$, 537.1 $(M - 5H + Na)^{4-}$, 531.6 M $- 4H)^{4-}$; estimated molecular mass 2130.7, theoretical molecular mass for $C_{69}H_{92}O_{43}N_{24}P_6$ 2131.46; analytical HPLC (Partisphere SAX column, 10 × 0.5 cm, 5 μm, Whatman; A, 0.01 M NaH_2PO_4 in 3:7 ACN/H_2O, pH = 6.4; B, 0.2 M NaH_2PO_4 in 3:7 ACN/H_2O, pH = 6.4; linear gradient from 0 to 100% of B in 20 min) t_R 12 min (Figure 6, chromatogram e).

Stability of Compound 9. Aliquots (100 μL) of a solution of peptide–oligonucleotide conjugate **9** (*ca.* 0.5 OD_{260} units) were treated with the following reagents: (i) 1 M aqueous piperidine, 22 h, rt; (ii) 1 M aqueous piperidine, 15 h, 55 °C; (iii) 0.05 M K_2CO_3 in 1:1 MeOH/dioxane, 6 h, rt; and (iv) 0.25 M LiOH in 1.5:1.5:1 MeOH/dioxane/H_2O, 6 h, rt. The product was found to remain stable under all these conditions, as controlled by anion exchange analytical HPLC: Partisphere SAX column (Whatman, 10 × 0.5 cm, 5 μm) A, 0.01 M NaH_2PO_4, pH = 6.4 in 3:7 ACN/H_2O; B, 0.2 M NaH_2PO_4, pH = 6.4 in 3:7 ACN/H_2O; linear gradient from 0 to 100% of B in 20 min; t_R of **9** 11.4 min. **9** was also treated with 80% AcOH at rt and at 55 °C, and its evolution was checked by HPLC under the same anion exchange analysis conditions; 33% of **9** was degraded to the oligonucleotide 5′-phosphate after 22 h at room temperature, and 28% and quantitative degradation were found, respectively, after 1 and 15 h at 55 °C (t_R of $p^{5'}$-CATCAT 14.7 min).

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Supporting Information Available: ^{1}H-decoupled ^{13}C-NMR spectra of **3** (1 page). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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