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Stepwise Solid-Phase Synthesis of Nucleopeptide Phac-Ser(p5'CATCAT)-Gly-Asp-OH from Adenovirus-2 Nucleoprotein

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Abstract. Nucleopeptide Phac-Ser(p5'CATCAT)-Gly-Asp-OH, corresponding to the linking site of adenovirus-2 nucleoprotein, has been synthesised by a stepwise solid-phase methodology. After assembly of the peptide on a solid support, the oligonucleotide chain elongation was carried out at the hydroxyl group of the serine residue by the phosphite triester approach using 5'-phosphoramidite derivatives. The target nucleopeptide was then obtained in good yield after removal of the permanent protecting groups under mild basic conditions.

Peptides and oligodeoxynucleotides can, in principle, be routinely assembled on a solid matrix using reliable standard methodologies, and in each case the synthesis cycles have many parallel steps for both types of molecules: removal of the temporary α-amine/5'-hydroxyl protecting group, coupling of the following monomer and blocking of unreacted groups on the polymer. However, the synthesis of nucleopeptides (hybrid molecules with a phosphodiester bond between the side chain hydroxyl group of an amino acid and the terminal hydroxyl group of an oligonucleotide) is a challenging undertaking which, to date, has not been resolved in a simple way despite the many efforts involved and the successful preparation of nucleopeptides of a certain complexity[1,2]. The critical point in the overall strategy is the protection scheme, which has to be designed in order to reduce the final cleavage and deprotection steps to a minimum but, more importantly, taking into account the stability of the target molecule. In this respect, it is well known that strong acid media can cause cleavage of the purine-deoxyribose bond and that the serine-nucleoside phosphodiester linkage is rather labile to bases[3,4].

We have recently described[5] the first example of the use of a solid-phase stepwise approach for the assembly of nucleopeptide Phac-Phe-Val-Ser(p3'ACT)-Gly-OH[6], where a phosphate diester links the peptide and the 3' end of the oligonucleotide. We now wish to report on the stepwise solid-phase synthesis of the nucleopeptide Phac-Ser(p5'CATCAT)-Gly-Asp-OH, **3**, which is part of the linking site of adenovirus-2 nucleoprotein[7]. The phosphodiester linkage here is established with the 5' end of the oligonucleotide chain and the molecule contains the trifunctional aspartic acid residue.

With respect to the protection sheme, we have used permanent protecting groups that can be removed under mild basic conditions (see below) and acid-labile Boc and DMT groups for the temporary protection of both the α-amine and the 3'-hydroxyl groups, respectively. For the preparation of the target nucleopeptide (Figure 1), the tripeptide was first assembled on the appropriately functionalised resin, and the oligonucleotide elongation was then carried out at the unprotected side chain hydroxyl group of the serine residue by the phosphite triester approach using 5'-deoxyribonucleoside phosphoramidite derivatives. Since the repetitive

strongest acid treatments required to deprotect the α-amine group (30-40% trifluoroacetic acid in dichloromethane) are carried out during the peptide assembly, there is no risk of depurination of the oligonucleotide part of the molecule.

The C-terminal amino acid was attached to a polystyrene solid support through a 2-(*o*-nitrophenyl)ethyl ester linkage[8-10] and the tripeptide elongation was carried out with commercially available N^{α}-Boc-amino acids. The β-carboxy function of aspartic acid was protected as a 9-fluorenylmethyl ester and Boc-serine was incorporated without protection of the hydroxyl group. Once the peptide was assembled, the N-terminus was blocked by reaction with phenylacetic acid in order to facilitate the NMR analysis of the final nucleopeptide.

The oligonucleotide chain was assembled at the serine hydroxyl group by standard oligonucleotide synthesis methodology with the minor modifications which make it compatible with a polystyrene matrix[11,12]. 5'-Phosphoramidite derivatives were activated and coupled in the presence of 5-(*o*-nitrophenyl)tetrazole. Once again, we chose permanent protecting groups which may be removed under mild basic conditions: dimethylformamidine[13-15] and isobutyryl[16,17] groups to protect the exocyclic amines of dA and dC, respectively, and 2-cyanoethyl groups for the phosphate functions. 3'-O-DMT-deoxynucleoside-5'-phosphoramidites with the above indicated nucleobase protecting groups are not commercially available and were specially prepared for this purpose. dA^{Dmf} was synthesised by reaction of dA with the dimethylacetal of N,N-dimethylformamide in methanol, and dC^{iBu} was obtained after transient trimethylsilylation of the hydroxyl groups and reaction with isobutyryl chloride in pyridine. $iPr_2N(CNEO)P\text{-}O^{5'}\text{-}dNu\text{-}^{3'}O\text{-}DMT$ (dNu= dA^{Dmf}, dC^{iBu} and T) were synthesised by the following procedure: i) the 5'-hydroxyl group was protected as a *tert*-butyldimethylsilyl ether by reaction with *tert*-butyldimethylsilyl chloride in pyridine; ii) the DMT group was introduced into the 3' position with dimethoxytrityl chloride in pyridine; iii) the silyl group was removed by a treatment with tetrabutylammonium fluoride in tetrahydrofuran, and iv) the 5'-phosphoramidite derivatives were obtained by phosphitylation with $CNEO\text{-}P(NiPr_2)_2$ in the presence of tetrazole[18].

The target nucleopeptide was obtained after a two-step deprotection process: a treatment with 0.05 M tetrabutylammonium in tetrahydrofuran (60 min), which cleaves the nucleopeptide-resin bond by β-elimination and removes the fluorenylmethyl and cyanoethyl groups, followed by an overnight treatment with conc. aq. ammonia/dioxane 1:1 at room temperature to eliminate the base protecting groups. The product was purified[19] by ion-exchange chromatography on DEAE-Sephadex and C18 reversed-phase MPLC, and was obtained in 33% overall synthesis, deprotection and purification yield.

Nucleopeptide **3** was shown to be homogeneous by HPLC[20] and different from the oligonucleotide-phosphate which would be formed if it were degraded by β-elimination[21]. Characterisation was accomplished by amino acid analysis after acid hydrolysis[22], nucleoside composition after digestion with snake venom phosphodiesterase and alkaline phosphatase[23], and ^{1}H- and ^{31}P-NMR[24]. Finally, the integrity of the molecule was definitively confirmed by electrospray mass spectrometry[25].

In summary, the solid-phase synthesis of a nucleopeptide with a phosphodiester linkage between a hydroxyl group in a peptide and the 5' end of an oligonucleotide, as in the nucleoproteins associated with the replication of the genetic material of some viruses[26], has been carried out by a stepwise approach for the first time. This result confirms that this methodology, much simpler than other previously reported and already shown to be convenient for the preparation of 3'-nucleopeptides, is of general application and can be successfully used to obtain nucleopeptides with a peptide linked to either end of the oligonucleotide chain.

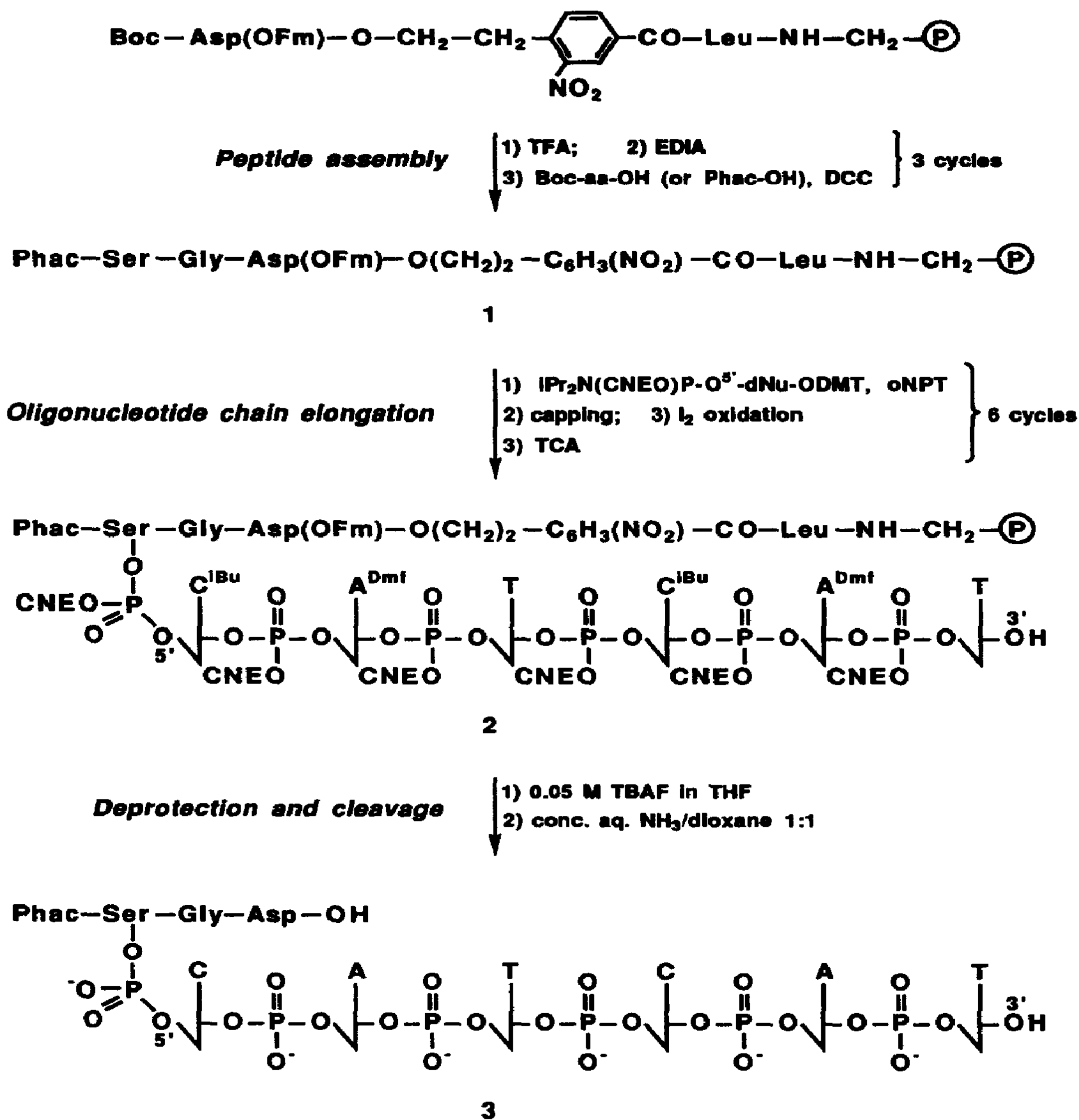

Figure 1. Synthesis of the nucleopeptide Phac-Ser(p5'CATCAT)-Gly-Asp-OH.

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6. Abbreviations: ACN=acetonitrile; Boc=*tert*-butoxycarbonyl; CNE=2-cyanoethyl; DCC=N,N-dicyclohexylcarbodiimide; Dmf=dimethylformamidine; DMT=4,4'-dimethoxytrityl; dNu=deoxynucleoside; EDIA=ethyldiisopropylamine; Fm=9-fluorenylmethyl; iBu=isobutyryl; MPLC= medium pressure liquid chromatography; oNPT=5-(*o*-nitrophenyl)tetrazole; P=polystyrene-*copoly*-divinylbezene; Phac= phenylacetyl; TBAF=tetrabutylammonium fluoride; TCA=trichloroacetic acid; TFA=trifluoroacetic acid; THF=tetrahydrofuran.
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10. 4-hydroxyethyl-3-nitrobenzoic acid[8] was coupled onto leucylaminomethyl-polystyrene (leucine was used as internal reference amino acid) in the presence of DCC and 1-hydroxybenzotriazole, and the C-terminal amino acid, Boc-Asp(OFm)-OH, was linked to the solid matrix by reaction with DCC and DMAP. Unreacted resin-bound amines were acetylated prior to the stepwise elongation of the peptide.
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18. ^{31}P-NMR: $iPr_2N(CNEO)P\text{-}O^{5'}\text{-}dA^{Dmf}\text{-}^{3'}O\text{-}DMT$, δ 148.5, 148.0 ppm; $iPr_2N(OCNE)P\text{-}O^{5'}\text{-}dC^{iBu}\text{-}^{3'}O\text{-}DMT$, δ 149.9, 147.5 ppm; $iPr_2N(CNEO)P\text{-}O^{5'}\text{-}T\text{-}^{3'}O\text{-}DMT$, δ 148.6, 147.9 ppm.
19. DEAE-Sephadex: gradient of triethylammonium bicarbonate, from 0.2 to 1M; MPLC (Vydac, C18) conditions: A: triethylammonium acetate 0.05 M, B: ACN/H_2O 1:1, gradient from 10 to 25% of B, detection of the elution profile at 254 nm.
20. Analytical HPLC: i) Spherisorb-ODS, the same eluents as for the MPLC, gradient from 15 to 21% of B in 20 min, 1 mL/min, detection wavelength: 260 nm, t_R : 11.1 min; ii) SAX (Partisphere), A: 0.01 M NaH_2PO_4 pH=6.4/ACN 7:3, B: 0.2 M NaH_2PO_4 pH=6.4/ACN 7:3, gradient from 0 to 100% of B in 20 min, 1 mL/min, detection wavelength: 260 nm, t_R: 11.9 min.
21. HPLC analysis of phosphate-oligonucleotide under anion-exchange conditions (see ii in reference 20), t_R: 13.5 min.
22. Amino acid composition (hydrolysis with 12 M HCl/propionic acid 1/1, 90 min, 150°C): Asp 1.0, Ser 0.6, Gly 1.0. The serine residue is partially destroyed under these hydrolysis conditions.
23. Nucleoside composition: dC 1.1, T 0.9, dA 1.0. The product remains undegraded after digestion with calf spleen phosphodiesterase.
24. ^{31}P-NMR: δ 2.0 and 3.1 ppm (approx. 5:1).
25. Electrospray MS ($C_{75}H_{95}O_{44}N_{23}P_6$, M=2208.6): m/z 441.0 $[M\text{-}5H]^{5-}$, 445.0 $[M\text{-}6H+Na]^{5-}$, 551.4 $[M\text{-}4H]^{4-}$, 557.3 $[M\text{-}5H+Na]^{4-}$, 735.3 $[M\text{-}3H]^{3-}$, 742.7 $[M\text{-}4H+Na]^{3-}$.'
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