Pergamon

Tetrahedron 58 (2002) 6965–6978

TETRAHEDRON

Towards nucleopeptides containing any trifunctional amino acid (II)

Laurent Debéthune, Vicente Marchán, Gemma Fàbregas, Enrique Pedroso and Anna Grandas*

Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Received 14 March 2002; revised 7 May 2002; accepted 18 June 2002

Abstract—Nucleopeptides with no restriction in the amino acid composition can be synthesized using stepwise solid-phase methodology. Best conditions for the protection of arginine and cysteine, as well as for the final deprotection treatment are established, and our conclusions can be extended to the preparation of any peptide–oligonucleotide hybrid. The association between certain side reactions and nucleopeptide sequences is also discussed. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Phosphodiester linkages between the side chain of hydroxylated amino acids and the ends of oligonucleotide chains are established during the replication of the genetic material of some viruses[1] and the topoisomerase-mediated relaxation of supercoiled DNA.[2] The small hybrid molecules that reproduce this particular type of amino acid–nucleoside covalent union are referred to as nucleopeptides. Nucleopeptides may be thus used as models of such nucleoproteins and offer great potential for the development of new antiviral and anticancer drugs.

The covalent attachment of a peptide to an oligonucleotide chain has been exploited[3] to facilitate the oligonucleotide transport through cell membranes and compartments, to strengthen the hybridization between two polyanionic DNA chains, to obtain artificial nucleases, and to introduce reporter groups.[4] Peptide–oligonucleotide hybrids, either with a phosphodiester or with other types of covalent linkage, can also be used for the study of DNA–protein interactions, to investigate the molecular requirements for enzyme activity,[5] and to evaluate how metals or metal-based anticancer drugs like cisplatin behave when DNA and proteins are in close proximity.[6–8] Finally, conjugation to the 3′ end delays oligonucleotide degradation by the most ubiquitous 3′ exonucleases.[9,10]

Our research group has been involved, over the last years, in the development of a methodology for the preparation of nucleopeptides. Our aim has been to set up a simple procedure to allow any nucleoside or amino acid to be introduced in the hybrid molecule, which required to find a suitable synthetic strategy and an appropriate set of permanent protecting groups. The stepwise solid-phase methodology was more appropriate than a convergent solid-phase approach.[11] Although the latter was useful in some cases,[10–20] we have found problems associated with the insolubility of protected peptides, in particular during the phosphitylation and coupling steps, and the target nucleopeptide has not always been obtained. The insolubility of some protected peptides may also render unsuitable the solution phase convergent approaches used for the preparation of certain peptide–oligonucleotide conjugates.[21–23] Stepwise oligonucleotide elongation (phosphite triester approach) at the side chain of a hydroxylated amino acid on a peptide-resin proceeds smoothly, whereas the subsequent incorporation of amino acids onto linker-modified oligonucleotide-resins does not give high, reproducible yields.[24] Regarding the protection scheme, nucleobases, phosphate groups, amino acid side chains, and the C-terminal carboxyl group (linkage to the solid support) were permanently protected with base-labile groups, whereas the temporary protection of 5′-hydroxyls and N^{α}-amines was achieved by acid-labile groups.

In previous reports,[11,24–26] we have described the synthesis of nucleopeptides incorporating all deoxyribonucleotides and, in addition to the linking residue, amino acids with most side chain functional groups (aspartic acid, histidine, methionine, serine, threonine, tyrosine, lysine, arginine, and tryptophan). This manuscript describes our latest results on the incorporation and protection of arginine, asparagine (or glutamine), and cysteine (Table 1, nucleopeptides **1** to **10**). We also discuss the compatibility of the final basic deprotection conditions with some nucleopeptide sequences. This study completes our nucleopeptide synthesis project,

Keywords: nucleopeptide; arginine; asparagine; cysteine; protection.

* Corresponding author. Tel.: +34-93-4021263; fax: +34-93-3397878; e-mail: agrandas@qo.ub.es

PII: S0040-4020(02)00793-7

Table 1. Nucleopeptides prepared in this study

1	Ac-Tyr(p$^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH
2	Ac-Tyr(p$^{3'}$dTTTCAGAA)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH
3	Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-OH
4	Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-NH_2
5	H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH
6	H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-OH
9	Ac-Cys-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH
10	Ac-Cys-Gly-Ala-Hse(p$^{3'}$dGCATGC)-Ala-OH
11	Ac-Gly-Ser(p$^{3'}$$T_5$)-Leu-Ala-Val-OH
12	Ac-Gly-Thr(p$^{3'}$$T_5$)-Leu-Ala-Val-OH
13	Ac-Gly-Tyr(p$^{3'}$$T_5$)-Leu-Ala-Val-OH
14	Ac-Gly-Tyr(p$^{5'}$$T_5$)-Leu-Ala-Gly-OH

since the methodology for incorporating any amino acid into nucleopeptides is now established.

2. Results and discussion

2.1. Amino acids side chain protection

2.1.1. Arginine. Protection of the guanidine function with two Fmoc[†] groups was shown to be suitable for preparing nucleopeptides, but N^{α}-Boc-Arg(Fmoc)$_2$-OH was not easily obtained.[26] The arginine side chain was also protected with two Fmoc groups for the preparation of peptide–oligonucleotide hybrids.[27] Protected arginines were obtained by reaction of unprotected ornithine-containing peptide-resins with *N*,*N*′-di-Fmoc-guanidine-*N*″-triflate in this case.

To facilitate the preparation of peptide–oligonucleotide hybrids, we evaluated the protection of the guanidine group simply by protonation, which gives good results in peptide synthesis.[28,29] The nucleopeptides Ac-Tyr(p$^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH (**1**)[5] and Ac-Tyr(p$^{3'}$dTTTCAGAA)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH (**2**) were assembled (Fig. 1) using the two modes of protection.

The amino acid analyses of the two peptide-resins, Ac-Tyr-Leu-Asp(OFm)-Pro-Arg(H$^+$)-Ile-Thr(Ac)-Val-resin and Ac-Tyr-Leu-Asp(OFm)-Pro-Arg(Fmoc)$_2$-Ile-Thr(Ac)-Val-resin, gave similar results, and the HPLC traces of the peptide crudes obtained after ammonia deprotection (NH_3/dioxane 1:1, 4 h, rt) were also similar (Fig. 2). However, in the crude obtained from protonated arginine, the target peptide was accompanied by a minor side product, namely Ac-Tyr-Leu-Asp-Pro-Orn(Ac-Tyr-Leu)-Ile-Thr-Val-OH, as inferred from MALDI-TOF mass spectrometric analysis.

All nucleopeptide crudes contained the target products, as confirmed by MALDI-TOF MS analysis, but products with *m*/*z* ratios corresponding to Ac-Tyr(p$^{3'}$dTTTCAGAA)-Leu-Asp-Pro-Arg(p$^{3'}$dTTTCAG)-Ile-Thr-Val-OH and Ac-Tyr(p$^{3'}$dTTTCAGAA)-Leu-Asp-Pro-Arg(p$^{3'}$dTTTCAGA)-Ile-Thr-Val-OH were also detected when nucleopeptide **2** was assembled on Ac-Tyr-Leu-Asp(OFm)-Pro-Arg(H$^+$)-Ile-Thr(Ac)-Val-resin. Both HPLC and PAGE analysis after overnight deprotection with NH_3/dioxane 1:1 at 55°C showed that nucleopeptide crudes obtained using Arg(Fmoc)$_2$ were much more homogeneous than those obtained using Arg(H$^+$) (Fig. 2). Bands with an electrophoretic mobility lower than that of the target nucleopeptides, corresponding to products with approximately twice as many nucleotide units, were found in the crudes obtained from Arg(H$^+$).

Altogether, these results show that although protonation keeps guanidine mostly unreactive during peptide assembly, the use of Arg(Fmoc)$_2$ derivative is recommended for the stepwise solid-phase assembly of nucleopeptides, and, in general, of peptide–oligonucleotide hybrids. Arginine protection with two Fmoc groups was safer for the construction of both the peptide and the oligonucleotide, since it avoided some side reactions during the elongation of the two chains. Using the protonated derivative, arginine was partially transformed into ornithine[30] and acylated by some of the incoming amino acids, and nucleotide incorporation onto the guanidine group occurred during the subsequent nucleotide incorporation cycles.

2.1.2. Asparagine and glutamine. Primary carboxamides react with activated phosphines and phosphoramidites[31] (Fig. 3A). This reaction was exploited for the preparation of peptide–oligonucleotide conjugates,[31] nucleic acid analogues[32] and aminoacyl-adenylate[33] with *N*-acylphosphoramidate linkages. We inferred that nucleopeptide assembly without protection of the asparagine and glutamine side chains may afford branched molecules, and searched for a permanent protecting group for these amino acid side chains that could be removed in mild, basic conditions. We disregarded the alternative of replacing asparagine (or glutamine) by the phenyl ester derivative of aspartic (or glutamic) acid, which may afford asparagine or glutamine by ammonolysis, because ammonia treatment may give rise to the

[†] Abbreviations: amino acid and oligonucleotides nomenclature follows the IUPAC-IUB recommendations (*Eur. J. Biochem.* **1984**, *138*, 9–37 and *Pure Appl. Chem.* **1974**, 279–290); Aa=amino acid, Ac=acetyl, Acm=acetamidomethyl, Boc=*t*-butoxycarbonyl, Bz=benzoyl, cHex=cyclohexyl, CNE=2-cyanoethyl, DBU=1,8-diazabicyclo[5.4.0]undece-7-ene, DCC=*N*,*N*′-dicyclohexylcarbodiimide, DCM=dichloromethane, DIEA=*N*,*N*-diisopropylethylamine, Dmf=*N*,*N*-dimethylamino-methylene, DMAP=4-*N*,*N*-dimethylaminopyridine, DMT=4,4′-dimethoxytrityl, Dnp=2,4-dinitrophenyl, DTT=1,4-dithiothreitol, For=formyl, Fm=9-fluorenylmethyl, Fmoc=9-fluorenylmethoxy-carbonyl, HMFS=2-(9-oxymethylfluorenyl)succinyl, HOBt=1-hydroxybenzotriazole, iBu=isobutyryl, IRAA=internal reference amino acid, MBHA=4-methylbenzhydrylamine, N=2′-deoxynucleoside, NMI=*N*-methylimidazole, pr=protected/protecting group, TBAF=tetrabutylammonium fluoride, TCA=trichloroacetic acid, TFA=trifluoroacetic acid, Tfa=trifluoroacetyl, THAP=2,4,6-trihydroxyacetophenone, Tos=tosyl.

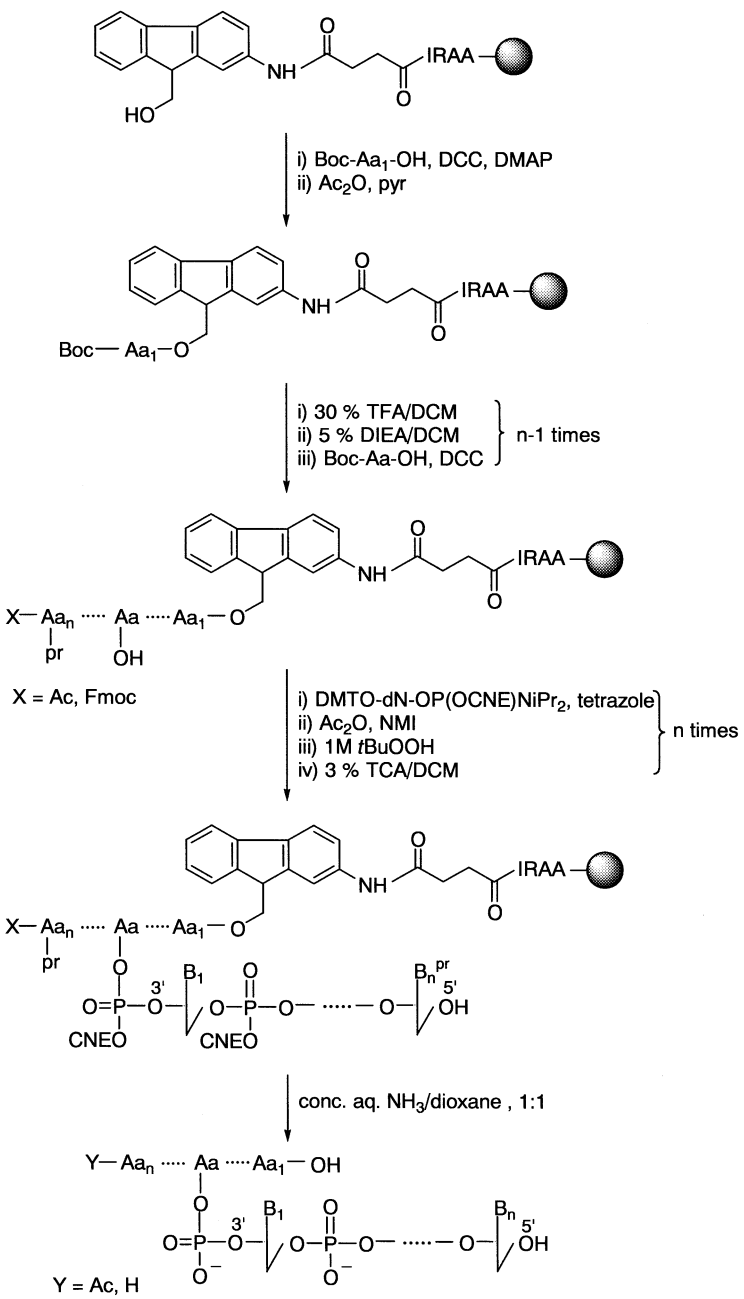

Figure 1. General nucleopeptide synthesis scheme.

formation of aspartimides to large extents.[34,35] In addition, both α- and β-peptide chains can be obtained from the reaction of ammonia with the aspartimide ring (Fig. 3B).

We first considered the use of protecting groups removable by β-elimination reactions, in particular the 2,4-dinitrophenylethyl group.[36] For preliminary evaluation, *N*-[β-(2,4-dinitrophenyl)ethyl]acetamide was obtained from *N*-[(β-phenyl)ethyl]acetamide by reaction with conc. H_2SO_4 and fuming HNO_3. We expected basic reagents to transform this product into acetamide and 2,4-dinitrophenylstyrene (Fig. 3C, a), but the starting material remained unaltered after a variety of treatments (overnight treatment with conc. aq. NH_3/H_2O 1:1 at rt or at 55°C; reaction with 0.5 M solutions of DBU in dioxane, EtOH/dioxane 1:1, pyridine or MeOH/pyridine 1:1, at rt for 24 h; and reaction with 0.1 M TBAF in anhydrous THF for 24 h at rt).

Attempts to oxidize $-CH_2-NH-$ groups to $-CO-NH-$ functions following published methods[37,38] also failed (Fig. 3C, b). This approach would have allowed protected 2,4-diaminobutyric acid and ornithine to be transformed into protected asparagine and glutamine, respectively. Efforts to protect the $CONH_2$ function with either benzoyl, diphenylcarbamoyl or Fmoc groups (Fig. 3C, c) were also unsuccessful.

After these negative results, we re-examined the extent of carboxamide phosphitylation on peptide-resins. Boc-Gly-Asn-Ala-Pro-MBHA, Boc-Gly-Gln-Ala-Pro-MBHA and Boc-Asn-Ala-Val-*O*-HMFS-Leu-MBHA were submitted

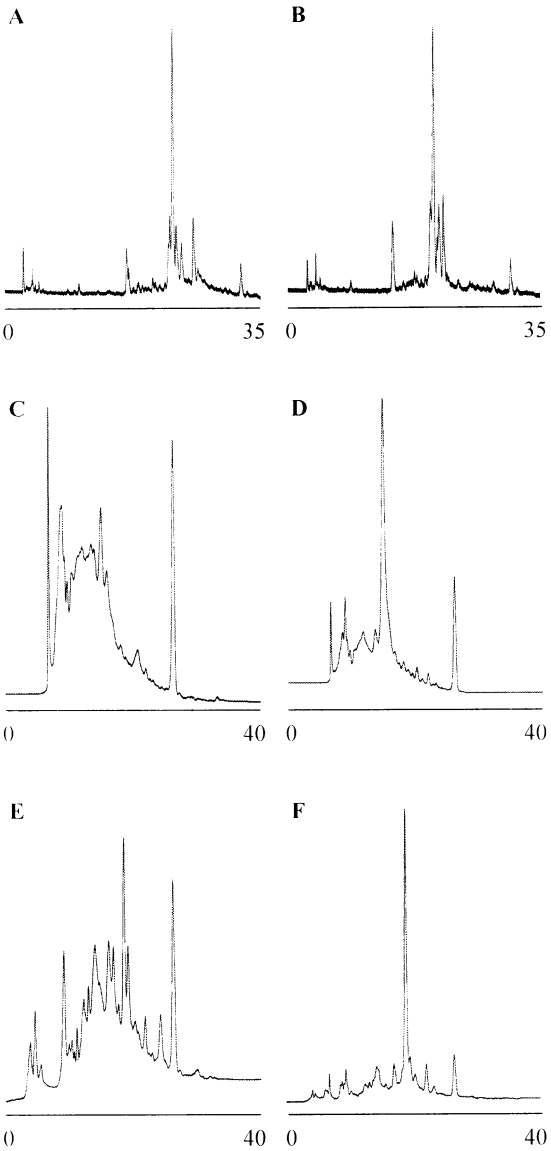

Figure 2. HPLC profiles of arginine-containing peptide and nucleopeptide crudes. Crudes obtained from protonated arginine: Ac-Tyr-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH (A), nucleopeptides **1** (C) and **2** (E). Crudes obtained from bis-Fmoc-protected arginine: peptide (B), nucleopeptides **1** (D) and **2** (F).

to four or five consecutive nucleotide (T) incorporation cycles, and the amount of DMT^+ formed after each cycle was quantitated. It appeared that some nucleoside incorporation had taken place during the first cycle (4–10% with respect to phosphitylation of all carboxamide groups on the solid matrix), but the amount of DMT^+ groups found after the subsequent synthesis cycles differed between assays. Nucleoside incorporation seemed to remain at ca. 1% in each of the subsequent cycles. No differences were found between asparagine and glutamine on the peptide-resin. MALDI-TOF analysis of the crude obtained after ammonia treatment of Boc-Asn-Ala-Val-*O*-HMFS-Leu-MBHA afforded peaks with *m/z* values corresponding to Boc-Asn(p$^{3'}$T)-Ala-Pro-OH, Boc-Asn(p$^{3'}$TT)-Ala-Pro-OH, Boc-Asn(p$^{3'}$TTT)-Ala-Pro-OH and Boc-Asn(p$^{3'}$TTTT)-Ala-Pro-OH in addition to tripeptide Boc-Asn-Ala-Pro-OH. However, the HPLC traces showed that the proportion of each of the side products in the peptide crude was very low (less than 3%).

As a final test, four nucleotide (T) incorporation cycles were carried out on a peptide-resin suitable for nucleopeptide synthesis, Ac-Asn-Phe-Val-Ser-Gly-*O*-HMFS-Leu-MBHA, which contained free hydroxyl and carboxamide groups. HPLC Analysis showed that the target nucleopeptide was the main product (75%) in the crude obtained after ammonolysis at rt (a mixture of C-terminal acid, Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-OH, **3**, and carboxamide, Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-NH_2, **4**, was obtained, see below). The only side product detected in substantial amount (ca. 25%) was the phosphorylated tetranucleotide TpTpTpTp, present in the mixture as the result of a β-elimination process that cleaves the nucleopeptide (see below).

Although there is a risk of obtaining branched products when the side chains of asparagine and glutamine are left unprotected, the extent of carboxamide phosphitylation by tetrazole-activated phosphoramidites seems to remain low and acceptable, unless stronger activators are used.[33] Hence, asparagine and glutamine protection is not essential for the standard stepwise solid-phase assembly of nucleopeptides (or of differently linked peptide–oligonucleotide hybrids), as reported elsewhere for phosphitylation of glutamine-containing peptide-resins.[39]

Nucleopeptides H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH (**5**)[5] and H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dTTTCAGAAAATC-TAG)-Leu-Asp-Pro-OH (**6**)[5] were then prepared by stepwise solid-phase assembly leaving unprotected the asparagine side chain.

Before oligonucleotide elongation, an aliquot of peptide-resin Fmoc-Ile-Ala-Leu-Gly-Thr(Ac)-Ser(Ac)-Lys(Tfa)-Leu-Asn-Tyr-Leu-Asp(OFm)-Pro-*O*-HMFS-Phe-MBHA was treated with the 1:1 conc. aq. NH_3/dioxane mixture at rt (overnight reaction). The target peptide was the main product of the crude, as inferred from MALDI-TOF MS and HPLC analysis (Fig. 4A). The subsequent incorporation of the required nucleoside derivatives (A^{Bz}, C^{Bz}, G^{iBu} and T) onto this peptide-resin yielded **5** and **6** after overnight deprotection and cleavage with conc. aq. NH_3/dioxane 1:1 at 55°C.

Analysis of the composition of the crude obtained from Fmoc-Ile-Ala-Leu-Gly-Thr(Ac)-Ser(Ac)-Lys(Tfa)-Leu-Asn-Tyr(p$^{3'}$dACGT)^{pr}-Leu-Asp(OFm)-Pro-*O*-HMFS-Phe-MBHA provided useful information. The target nucleopeptide, **5** (30%; MALDI-TOF MS, negative mode: *m/z* 2639.0), was accompanied by H-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH (**7**, 20%; MALDI-TOF MS, negative mode: *m/z* 1740.6) and a second product (**8**, 30%) with an *m/z* ratio virtually identical to that of **5** (*m/z* 2639.2, MALDI-TOF MS, negative mode) (Fig. 4B). Compound **5** was also identified by nucleoside and amino acid composition after enzymatic digestion. Amino acid analysis after proteolytic digestion confirmed the presence of both asparagine and aspartic acid. The digestion of **5** by a mixture of proteolytic

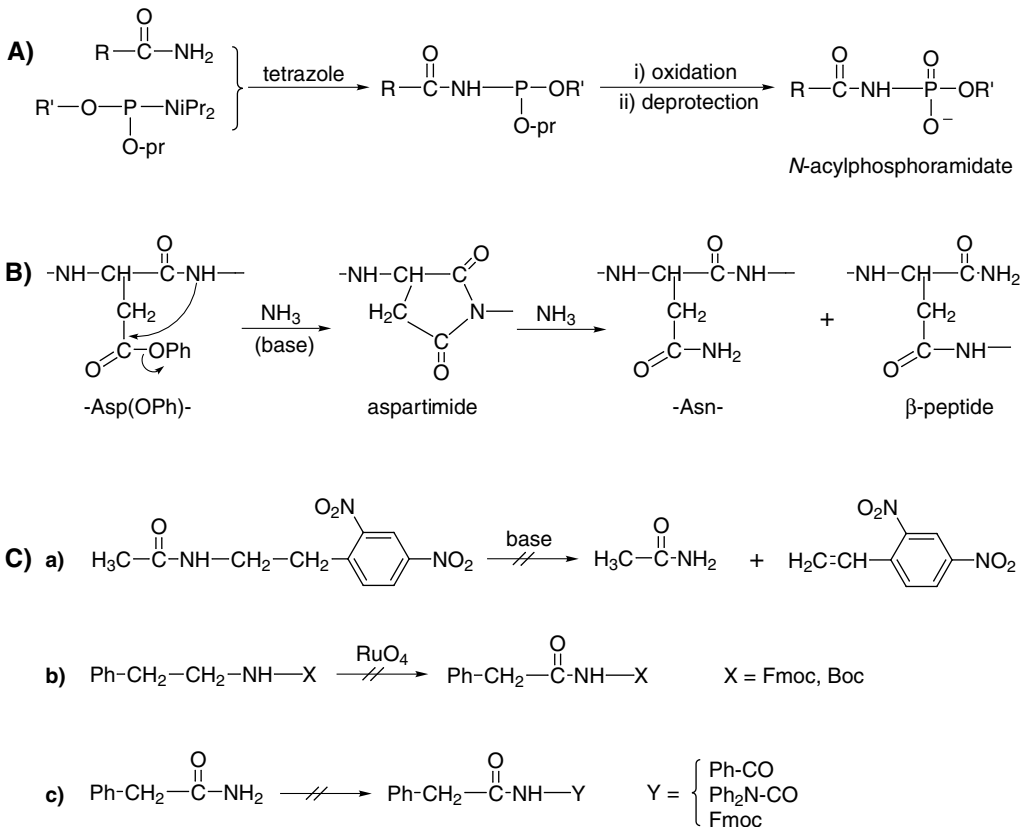

Figure 3. Side reactions associated with Asn (A) or protected aspartic acid (B), and summary of the essays carried out to obtain suitably protected carboxamide derivatives (C).

enzymes was the expected behavior for an α-peptide chain (see below).

Concerning the formation of the two side products, **7** seemed to result from the cleavage of the Asn-Tyr peptide bond, as a result of the nucleophilic attack of the side chain primary carboxamide to the asparagine carbonyl group of the Asn-Tyr union. Peptide cleavage is unlikely,[40] but intramolecular acylations leading to the formation of aspartimides are readily promoted by bases.

Side product **8** was formed from both the protected and the free, pure nucleopeptide upon ammonia treatment at 55°C, and it was shown to remain stable in these reaction conditions. An impurity with a mass virtually identical to that of the target product was also found to accompany **6**, as inferred from the MALDI-TOF MS analysis of the crude.

Compound **8** may be a nucleopeptide containing aspartic acid instead of asparagine. The deamidation reaction is enhanced by increased pH, ionic strength and temperature.[41] Since carboxylic acids do not react with ammonia to give amides, **8** cannot be transformed into **5**, which is consistent with its stability in these reaction conditions. Alternatively, the origin of **8** may be the nucleophilic attack of the NH of the Asn-Tyr amide bond to the side chain carboxamide, with the concomitant formation of aspartimide, followed by basic hydrolytic opening of the ring (Fig. 3C). Opening of the aspartimide ring by reaction with ammonia may yield both the target nucleopeptide and the β-peptide isomer. Formation of the latter was also consistent with the mass found for **8**. Nevertheless, **8** was stable to treatment with NH_3 at 55°C, supporting the first hypothesis. The primary carboxamide of the β-peptide may again react intramolecularly to give the aspartimide, which, in turn, may yield an [α-+β-] nucleopeptides mixture. In other words, if **8** were the β-peptide, it might be partially transformed into **5** in these reaction conditions, which did not happen.

The main conclusion from this study concerns the final deprotection conditions. Peptide H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr-Leu-Asp-Pro-OH and nucleopeptides Ac-Asn-Phe-Val-Ser($p^{3'}$TTTT)-Gly-OH/NH_2 (**3**+**4**) were the main products after ammonolysis at rt, whereas ammonia treatment of the protected precursors of **5** and **6** at 55°C afforded complex reaction mixtures. Moreover, pure **5** was partially transformed into the other two products after a 5 h treatment with conc. aq. NH_3/dioxane 1:1 at 55°C. Therefore, the use of the most base-labile set of permanent protecting groups to allow the final ammonia deprotection at rt is preferable, especially when asparagine is present in the peptide chain.

2.1.3. Cysteine. At this point, the only trifunctional amino acid that remained to be introduced in nucleopeptides was cysteine. However, we needed a protecting group able to prevent sulfur oxidation (and nucleotide coupling onto the thiol group) during nucleotide incorporation cycles. Only three commercially available Boc-cysteine derivatives were protected with groups removable in conditions compatible with a nucleopeptide structure, Boc-Cys(Fm)-OH, Boc-Cys(Acm)-OH and Boc-Cys(*S*–*t*Bu)-OH. The latter

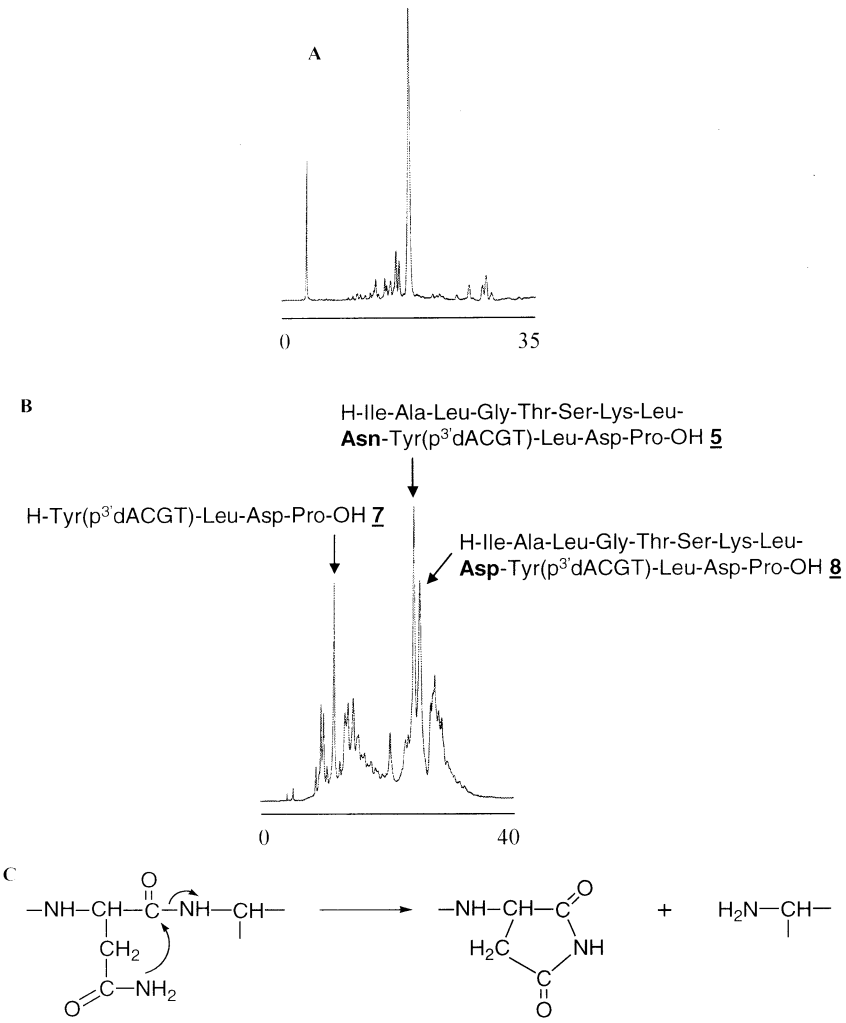

Figure 4. HPLC traces of crude H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr-Leu-Asp-Pro-OH (A) and H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH (**5**) (B). Possible mechanism accounting for the formation of side product **7** (C).

was the only one stable in the phosphite to phosphate oxidation conditions (both aqueous iodine and *t*-butylhydroperoxide, TLC analysis).

Thiol groups have been protected as disulfides, with the *S*–*tert*-butyl group, for the preparation of peptide–oligonucleotide conjugates either containing a cysteine residue[42,43] or linked through N^4-mercaptoethylcytosine.[44] Standard oxidation conditions were used during oligonucleotide elongation and the *S*–*tert*-butyl group was removed by reaction with conc. aq. NH_3 solutions containing DTT. The *S*–*tert*-butyl group is not labile in the ammonia deprotection conditions, but Schwope and co-workers[43] reported that, during this treatment, the SS*t*Bu group is lost by a β-elimination reaction, giving rise to dehydroalanine at the cysteine position. Dehydroalanine may be subsequently transformed into serine (or 2,3-diaminopropionic acid) by conjugate addition of OH^- (or NH_3) during the concentrated aqueous ammonia treatment (Fig. 5A).

Two cysteine-containing nucleopeptides were synthesized, Ac-Cys-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH (**9**) and Ac-Cys-Gly-Ala-Hse(p$^{3'}$dGCATGC)-Ala-OH (**10**). Both were assembled using standard stepwise solid-phase protocols, with two differences: (i) Ac-Cys-(*S*–*t*Bu)-OH was used for the synthesis of **9**, while **10** was prepared using Boc-Cys-(*S*–*t*Bu)-OH; (ii) standard phosphoramidite derivatives were used to obtain **9**, but, as deduced from the synthesis of **9** (see below), **10** was assembled using nucleoside derivatives protected with more base-labile groups (A^{Bz}, C^{Ac}, G^{Dmf}).

Regarding the deprotection of peptide-resins (Fig. 5B, a), the thiol-protected peptides (Ac-Cys(*S*–*t*Bu)-Gly-Tyr-Pro-OH and Ac-Cys(*S*–*t*Bu)-Gly-Ala-Hse-Ala-OH) were the main products of the respective crudes when deprotection was carried out with NH_3/dioxane 1:1 at rt (overnight treatment). The serine-containing peptide was the main product when the overnight ammonolysis reaction was carried out at 55°C, and the free peptide was obtained after cleavage and deprotection with the 1:1 mixture NH_3/[1 M DTT in dioxane] for 4 h at rt.

Most of the deprotection assays on nucleopeptide-resins were carried out with the precursor of **9**, and essentially reproduced the results obtained with peptide-resins (Fig. 5B, b). The target nucleopeptide was obtained as the main product after ammonia/DTT treatments at rt or for 7 h at 55°C. However, when the deprotection reaction was prolonged overnight at 55°C, the main product was a

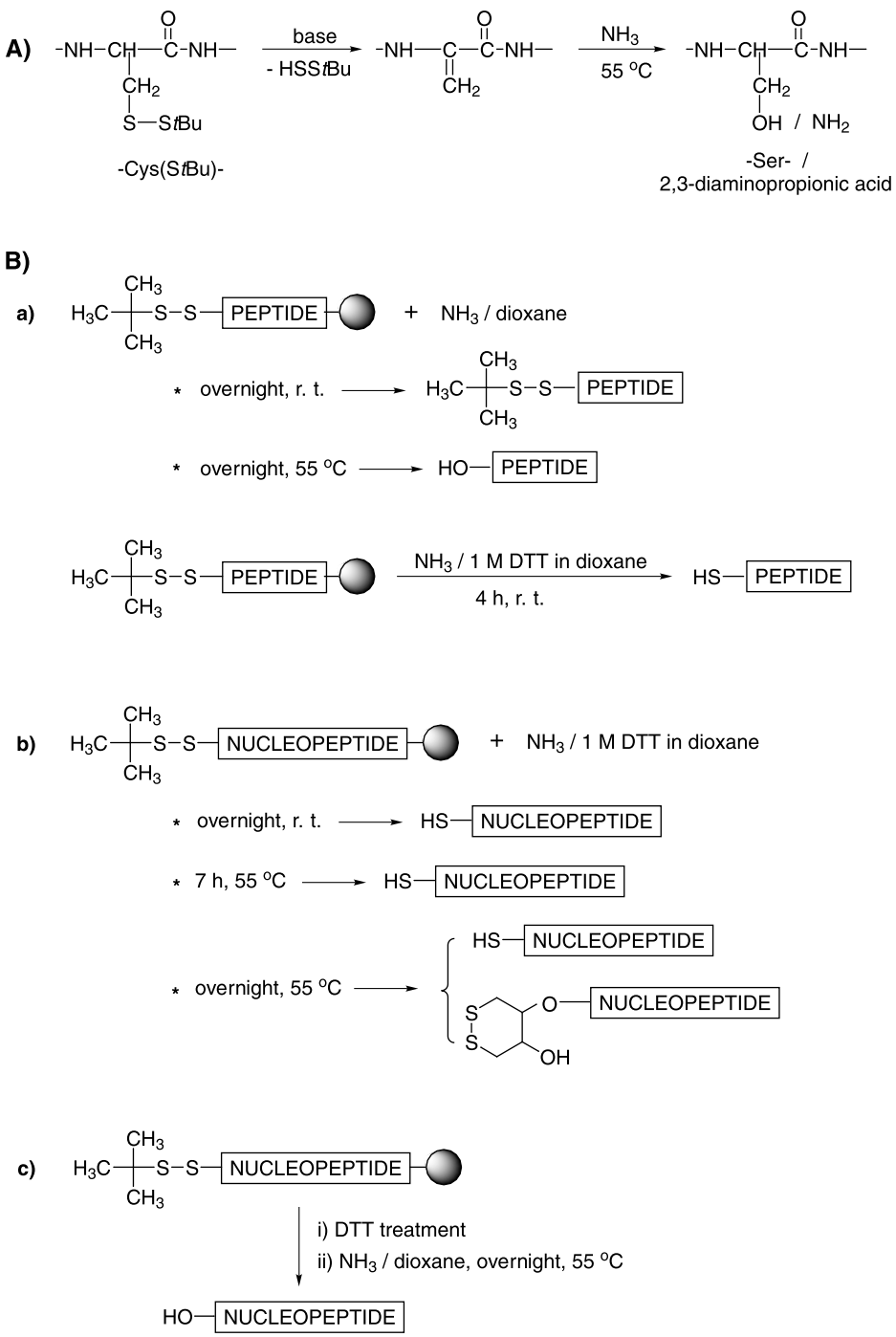

Figure 5. Side reactions associated with the deprotection of cysteine-containing peptides or nucleopeptides, and products obtained.

modified nucleopeptide, resulting from β-elimination and DTT addition to dehydroalanine.

In another experiment, we aimed to selectively remove the cysteine protecting group from the nucleopeptide-resin, since β-elimination does not occur if a cysteine-free nucleopeptide is treated with ammonia. In addition, this procedure was attractive because it avoided the presence of DTT in the nucleopeptide crude. Unfortunately, this experiment failed (Fig. 5B, c). Nucleopeptide Ac-Ser-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH was the main product, as expected for a base-treated Cys(*S*–*t*Bu)-containing nucleopeptide, when the nucleopeptide-resin was first submitted to treatment with 1 M DTT (1 h), either in dioxane or in dioxane/Tris HCl at pH=8, and then to reaction with NH_3/dioxane 1:1 at 55°C.

In conclusion, the best choice is to assemble cysteine-containing nucleopeptides using Cys(*S*–*t*Bu) in combination with the most base-labile nucleobase and amino acid side chain permanent protecting groups, to allow the mildest deprotection conditions (NH_3/[1 M DTT in dioxane] 1:1, 8 h to overnight treatment, rt).

For the isolation of **9**, the final cleavage and deprotection step was carried out by reaction with the NH_3/DTT mixture

for 7 h at 55°C, searching for nucleobase deprotection conditions compatible with the stability of the target molecule. After oxidation with aqueous dimethylsulfoxide,[45] we obtained the nucleopeptide dimer, with a disulfide bond linking the two units, {Ac-Cys-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH}$_2$[S–S] (as confirmed by analytical ultracentrifugation).

Unexpectedly, the isolation of **10** was much more difficult. Although the optimal protection scheme and deprotection conditions were used, a complex crude was obtained. As stated above, the same deprotection treatment at the level of peptide-resin had afforded the free peptide Ac-Cys-Gly-Ala-Hse-Ala-OH as the main product. As expected, the peptide cleanly dimerized to the disulfide form.

HPLC and MALDI-TOF MS analysis of the nucleopeptide crude showed that some of the contaminants were nucleopeptides lacking the Ac-Cys, Ac-Cys-Gly and Ac-Cys-Gly-Ala fragments. Moreover, pure **10**, in the reduced form, decomposed affording the same mixture of products and dimerized very slowly. The isolated nucleopeptide disulfide dimer {Ac-Cys-Gly-Ala-Hse(p$^{3'}$dGCATGC)-Ala-OH}$_2$[S–S] was stable. We cannot explain how the covalent linkage of an oligonucleotide to the homoserine side chain can give rise to a readily decomposing nucleopeptide, whereas peptide Ac-Cys-Gly-Ala-Hse-Ala-OH is stable.

We would also like to highlight that, in both nucleopeptide crudes, some *m*/*z* values corresponded to increases of 53 mass units with respect to the expected product. Alkylation of the free thiol groups by the acrylonitrile generated upon deprotection of the phosphates accounts for the formation of these [–S–CH$_2$–CH$_2$–CN]-modified side products. In agreement with other authors,[46] the proportion of these impurities was found to decrease when final deprotection was carried out in higher solvent volumes.

To gain more insight into the synthesis of cysteine-containing nucleopeptides, we checked the peptide stability in the oligonucleotide assembly conditions. A sample of Ac-Cys(*S*–*t*Bu)-Gly-Ala-Hse-Ala-resin was submitted to a nucleotide incorporation cycle, with no phosphoramidite delivery to the column to prevent coupling onto the homoserine side chain, and two aliquots were subsequently treated with either the NH$_3$/DTT mixture or with NH$_3$ (rt), respectively. The NH$_3$/DTT treatment afforded the free peptide. The NH$_3$ treatment gave rise to a complex mixture in which the expected thiol-protected peptide was a minor product, and which contained, among others, products with *m*/*z* ratios corresponding to oxidized peptides (the *m*/*z* ratios found indicated transformation of the S–*S*–*t*Bu moiety into SO$_2$–*S*–*t*Bu, and even to SO_3^- groups). On one hand, this experiment showed that, in contrast with the results of our initial tests, the S–*S*–*t*Bu moiety was not stable to oxidation with *t*BuOOH. These oxidized cysteines probably undergo β-elimination more easily than the unoxidized residues. On the other hand, the expected peptide was the main product when deprotection was carried out with the NH$_3$/DDT mixture, indicating that most, if not all, of these oxidized products were reduced by reaction with DTT during the deprotection process.

2.2. Stability of nucleopeptides in the final basic deprotection treatment conditions

The most delicate point of our stepwise solid-phase synthesis scheme is, as stated elsewhere,[26] the compatibility between the basic conditions used for the removal of the permanent protecting groups and the base-lability of serine- and threonine–nucleoside phosphate diester linkages.[47] Unfortunately, not all serine–nucleoside linkages have the same stability,[11] since, for instance, the first serine-nucleopeptide that we synthesized and used as model compound, Boc-Ser(p$^{5'}$dT)-NH-cHex, is the most base-stable nucleopeptide that we have ever obtained. We have found that: (i) some serine-nucleopeptides are extremely labile to bases and cannot be obtained even using the mildest deprotection conditions (1:1 NH$_3$/dioxane mixtures, rt), (ii) the extent of the β-elimination reaction is rather high in other cases, but (iii), it can be kept to acceptable limits (10–25%) in most situations. Examples of the first nucleopeptides (quantitative degradation after 2–6 h NH$_3$ treatments) are H-Ser(p$^{5'}$dG)-His-OH[44] and H-Ser(p$^{5'}$dG)-Met-OH.[24] Up to 60–70% degradation was found in the synthesis of Ac-Gly-Ala-Ser(p$^{3'}$dACTAGT)-His-Val-OH,[44] but the extent of the side reaction was lower for Ac-Gly-Ala-Ser(p$^{3'}$dACTAGT)-Lys-Val-OH (23%), which only differed from the previous molecule in that histidine was replaced by lysine.[48] So far, we cannot reach any conclusion relating the base-lability of nucleopeptides with their sequence. The -Ser-His- sequence may be very prone to undergo the β-elimination reaction, but no association between size and stability can be established.

Threonine-nucleopeptides are more stable to basic reagents than serine-nucleopeptides,[47] in agreement with our results. The extent of the β-elimination reaction after treatment of nucleopeptide-resin Ac-Gly-Ser(p$^{3'}$T$_5$)^{pr}-Leu-Ala-Val-*O*-HMFS-resin with a 1:1 mixture of NH$_3$/dioxane at rt was 32% after 6 h (as deduced, upon HPLC analysis, from the relative areas of the peaks corresponding to the target nucleopeptide, Ac-Gly-Ser(p$^{3'}$T$_5$)-Leu-Ala-Val-OH, **11**, and the phosphorylated tetranucleotide, TpTpTpTp). In the case of nucleopeptide Ac-Gly-Thr(p$^{3'}$T$_5$)-Leu-Ala-Val-OH, **12**, 23% degradation was detected after the same treatment. Other authors have described that threonine-nucleopeptides undergo the β-elimination reaction.[49]

As expected, with both linking amino acids, most of the side reaction occurs at the beginning of the basic treatment, before phosphate groups are fully deprotected, since phosphate diesters are much more efficient leaving groups than monoesters, and the prolonged reaction time required to fully remove the nucleobase permanent protecting groups does not excessively affect the target product. Protection of the phosphates and the nucleobases with allyl and allyloxycarbonyl groups, which can be removed in neutral conditions, has been claimed to be the best alternative to obtain serine-nucleopeptides, since only a short ammonia treatment is required to cleave the oligonucleotide–resin bond.[18,19] The disadvantage of this protection scheme is that nucleoside phosphoramidite derivatives are not commercially available.

Tyrosine gives rise to the most base-stable amino acid–nucleoside linkages, which are fully resistant to

NH_3/dioxane treatments.[26] No lability to tetrabutylammonium fluoride in aqueous solvents has been described,[50,51] but their stability to this reagent in anhydrous solutions has not been proved. The nucleopeptides Ac-Gly-Tyr($p^{3'}T_5$)-Leu-Ala-Val-OH (**13**) and Ac-Gly-Tyr($p^{5'}T_5$)-Leu-Ala-Gly-OH (**14**) were obtained by deprotection and cleavage of the nucleopeptide–resin bond with 0.05 M TBAF in anh. THF (30 min, rt), and no pentanucleotide was found upon HPLC analysis of the crudes. We thus conclude that tyrosine–nucleoside phosphodiester linkages are resistant to treatment with fluorides in anhydrous media.

2.3. C-Terminal acids vs C-terminal carboxamides

The NH_3/dioxane treatment used for the final deprotection of nucleopeptides is known to afford a mixture of nucleopeptides with the C-terminal amino acid either as carboxylic acid or as carboxamide derivative.[26,39] As expected, the relative proportion of these two products depends on the nature of the C-terminal amino acid (Table 2).

A C-terminal glycine residue always gives rise to acid/carboxamide mixtures, in a relative proportion which varies between nucleopeptides. In any case, β-elimination seems to take place faster than the ammonia nucleophilic attack to the carbonyl of the ester, since the acid is always obtained in higher yields than the amide. Alkaline hydrolysis of the ester may also account for the formation of nucleopeptide-acids.

Nucleopeptide Phac-Phe-Val-Ser($p^{3'}$dACTAGT)-Gly was also assembled using a TentaGel solid support instead of the polystyrene-1%-divinylbenzene copolymer. After cleavage and deprotection using the same conditions as described in Table 2, HPLC analysis of the crude revealed a 1.7:1 –OH/NH_2 mixture.[52] Should we rely on a single result to reach conclusions, this would indicate that the nucleophilic attack of ammonia to the carbonyl of the ester (and thus carboxamide formation) is slightly more favored with polystyrene resins, which have worse swelling properties in polar solvents than polystyrene–polyethyleneglycol copolymers.

Analysis of the last three nucleopeptide crudes (Table 2) showed that C-terminal amino acids with more sterically hindered side chains give rise, as expected, to considerably lower or undetectable amounts of carboxamide product. Nevertheless, the relative proportion of products probably varies depending on the structure (length, sequence) of the immobilized molecule, as in the case of our nucleopeptides with glycine at the C-terminus, since ammonia treatment of a peptide-HMFS-resin with a leucine residue at the C-terminal position has been described to yield a 3:1 –OH/–NH_2 mixture.[39]

Finally, we did not detect the formation of asparagine after ammonia deprotection of -Asp(OFm)- derivatives, which does not agree with the results found for C-terminal glycine nucleopeptides, but may be due to the proximity of the solid support. If the acidic proton of the fluorenylmethyl ester is less accessible, thus delaying the β-elimination reaction, nucleophilic attack of ammonia to the carbonyl of the ester may occur, obviously more easily if bulky groups are not close. Fluorenylmethyl groups on amino acid side chains are more accessible and may be smoothly removed, releasing the carboxyl group, whereas the solid matrix may hinder the elimination of the acidic proton of the fluorenylmethyl esters of C-terminal amino acids, thus enhancing the formation of acid/amide nucleopeptide mixtures.

3. Conclusion

Nucleopeptides, as well as other peptide–oligonucleotide conjugates, can be easily obtained by stepwise solid-phase synthesis. It is recommended to first assemble the peptide moiety, and then to elongate the oligonucleotide chain at the hydroxyl group of an amino acid side chain.

As often stated, the most delicate step is the choice of protecting groups. The use of base-labile permanent protecting groups is not devoid of problems, but, in our opinion, it is advantageous with respect to other alternatives because it allows the use of many commercially available amino acid and nucleoside synthons. Boc-Thr(Ac)-OH, Boc-Arg($Fmoc_2$)-OH and Boc-Cys(*S*-*t*Bu)-OH are the only amino acid derivatives that cannot be purchased from commercial sources. It may also be necessary to prepare the 5′-phosphoramidites of dC^{Ac} (or dC^{iBu}) and dG^{Dmf},[54–56] or

Table 2. Relative proportion nucleopeptide-OH/nucleopeptide-NH_2 after deprotection with ammonia

Nucleopeptide sequence	Deprotection conditions (NH_3/dioxane 1:1)	–OH/–NH_2
Phac-Phe-Val-Ser($p^{3'}$dACTAGT)-Gly[52]	17 h, rt	1.3:1
Ac-Ser($p^{5'}$dG)-Gly[47]	24 h, rt	1.7:1
Phac-Ser($p^{5'}$dG)-Gly[47]	24 h, rt	1.4:1
H-Trp-Val-Hse($p^{3'}$dACTAGT)-Gly[26]	17 h, 55°C	2:1
Phac-Hse($p^{3'}$dACTAGT)-Ala-Thr-Ser-Val-Tyr-Gly[26]	17 h, 55°C	2:1
Ac-Gly-Tyr($p^{3'}$TTTTT)-Leu-Ala-Gly	6 h, rt	3:1
	6 h, 55°C	4:1
Ac-Lys-Trp-Lys-Hse($p^{3'}$dGCATCG)-Ala[53]	17 h, 55°C	>93:7
Ac-Ala-Trp-Ala-Hse($p^{3'}$dGCATCG)-Ala[53]	17 h, 55°C	>93:7
Ac-Gly-Tyr($p^{3'}$TTTTT)-Leu-Ala-Val	6 h, rt	>99:1
	6 h, 55°C	>99:1

The relative proportion nucleopeptide-OH/nucleopeptide-NH_2 was deduced from the peak areas at the HPLC trace of each crude (the HFMS handle was used in all cases; the acid always eluted before the amide). The isolated products were characterized as the C-terminal acid and the C-terminal amide, respectively, by electrospray mass spectrometric analysis.

those in which nucleobases are protected with *tert*-butylphenoxyacetyl groups,[57] to obtain nucleopeptides with serine- or threonine–$^{5'}$oligonucleotide linkages, since the only 5′-phosphoramidite derivatives now available require deprotection with ammonia at 55°C.

We have obtained the best results by protecting the side chain of arginine with two Fmoc groups rather than by protonation. Guanidine protonation is insufficient to wholly prevent the formation of branched products resulting from nucleotide incorporation at this group, and less homogeneous crudes are obtained.

Protection of the primary carboxamides of asparagine and glutamine side chains is not mandatory, since the extent of phosphitylation of these groups is kept to minimal, acceptable levels during oligonucleotide elongation cycles. Ammonia deprotection at rt is required to avoid side reactions in asparagine-containing nucleopeptides.

The cysteine thiol can be blocked with the *tert*-butylsulfenyl group, which can be removed simultaneously to all the other permanent protecting groups by adding DTT to the final ammonia deprotection solution. We have found that, even in the presence of DTT, β-elimination of HS–S*t*Bu occurs when the ammonia treatment is carried out at 55°C, and that the extent of this side reaction grows with prolonged reaction times. Although no mention is made to this side reaction in some reports,[9,58] we recommend rt conditions for the final ammonia deprotection reaction. Large volumes of deprotecting solution are also helpful to minimize the extent of cysteine alkylation by acrylonitrile.

The base-lability of serine- and threonine–nucleoside linkages is one of the major points of concern, but final deprotection with ammonia at rt allows most nucleopeptides to be obtained in fairly good yields. Since mild deprotection conditions are also convenient to minimize or circumvent side reactions associated with the presence of asparagine and -Cys(*S*-*t*Bu), the use of the most base-labile nucleobase protecting groups is required for the stepwise synthesis of both nucleopeptides and any peptide–oligonucleotide conjugate incorporating cysteine or asparagine.

In summary, the most convenient scenario for nucleopeptide synthesis is that in which glycine is not the C-terminal amino acid, and either tyrosine or the natural, non-proteinogenic homoserine residue is the linking residue.[59] Anchoring of glycine to the solid support is racemization-free, but cleavage of the nucleopeptide–resin bond with ammonia yields acid/amide mixtures in unpredictable proportions. Although the mixture of the two products can be used in some biological experiments, the presence of two compounds renders purification and characterization more difficult. The only alternative to obtain a single product (the acid) is to detach the nucleopeptide from the solid matrix by reaction with tetrabutylammonium fluoride in anhydrous conditions, but this rather strong basic treatment may not be compatible with all nucleopeptide sequences. Regarding the base-stability of serine- and threonine–nucleoside phosphodiester linkages, on the basis of the few data available predictions cannot be made, and we have not found significant differences between the base-lability of 3′ and 5′-unions.

4. Experimental

Fmoc-L-amino acids, Boc-L-amino acids and resins for peptide synthesis (MBHA resin and Boc-Val-Pam-resin) were obtained from Bachem and Novabiochem. The 3′-phosphoramidite derivatives of DMT-dA^{Bz}, DMT-dC^{Bz}, DMT-dG^{iBu} and DMT-T, and the $^{5'}$ phosphoramidite derivative of T-DMT were purchased from Glen Research Corporation. Solid-phase syntheses were performed manually in a polypropylene syringe fitted with a polyethylene disc (peptides), or on the 380B Applied Biosystems synthesizer (oligonucleotide assembly in nucleopeptides).

HPLC analysis (Nucleosil C-18, 250×4.6 mm, 10 μm, 1 mL/min, detection wavelength 260 nm): A=0.01 M ammonium acetate, B=acetonitrile/H_2O 1:1 (v/v). Unless otherwise indicated, purifications at the semi-preparative scale were carried out on a Kromasil column (Phase Separations, 250×10 mm, 10 μm, 3 mL/min) using basically the same solvent system (A=0.05 M ammonium acetate).

Amino acid analyses were performed on a Beckman System 6300 analyzer. Acid hydrolyses of peptide-resins, peptides and nucleopeptides were carried out in 12 M HCl/propionic acid (1:1) either at 150°C for 60–90 min or at 110°C for 24 h (homoserine, serine and threonine are to some extent degraded in these conditions). Enzymatic hydrolyses of the peptide moiety of nucleopeptides were carried out as follows (the three enzymes were purchased from Sigma): to a solution of 3–5 nmol of product in 15 μL of 0.1 M ammonium acetate (pH=5.3) was added 1 μL of a 1:32 (v/v) 2-mercaptoethanol/H_2O mixture and 3 μL of a 20 mg/mL papaine (EC 3.4.22.2) solution (286 U/mL). The mixture was incubated at 37°C for 2 h, and, after addition of 1 drop of acetic acid, lyophilised. The sample was dissolved in 15 μL of 0.2 M ammonium acetate (pH=8.3) and 1 μL of the 2-mercaptoethanol/H_2O mixture. 3 μL of microsomal leucine aminopeptidase (EC 3.4.11.2) solution (3 mg/mL, 72 U/mL) was added, and the mixture was incubated at 37°C for 3 h. After a second addition of 3 μL of the enzyme solution, incubation was prolonged for 12 h (or overnight), 1 drop of acetic acid was added and the sample was lyophilized. Finally, 3 μL of a prolidase (EC 3.4.13.9) solution (5 mg/mL, 875 U/mL) was added to the amino acid/nucleopeptide mixture dissolved in 15 μL of 0.1 M ammonium acetate and 0.025 M $MnCl_2$ (pH=8.3). The mixture was incubated for 3 h at 37°C, after which 1 drop of acetic acid was added and the sample was lyophilized.

The nucleoside content in nucleopeptides was determined after reversed-phase HPLC analysis of the mixtures obtained after digestion with snake venom phosphodiesterase (EC 3.1.4.1, Sigma) and alkaline phosphatase (EC 3.1.3.1, Sigma).

Mass spectrometric analysis was carried out using

VG-Quattro (ES) or Applied Biosystems Voyager-DERP (MALDI-TOF) instruments.

4.1. Nucleopeptide synthesis

Incorporation of the internal reference amino acid onto *p*-methylbenzhydrylamine-resin (f=0.5–0.6 mmol/g) was carried out by reaction with 0.5 equiv. of both the corresponding Fmoc-amino acid derivative and DCC (15 min) in order to achieve partial acylation of polymeric amine groups. The substitution degree was determined on an aliquot by UV spectrophotometric quantitation of the amount of *N*-(9-fluorenylmethyl)piperidine formed upon deprotection with piperidine, and, if suitable for oligonucleotide synthesis,[60] unreacted amine groups were capped by reaction with Ac_2O and DIEA (substitution degree values ranged between 0.13 and 0.25 mmol/g). The Fmoc group was eliminated and the bifunctional linker *N*-2-(9-hydroxymethylfluorenyl)succinamic acid, H-HMFS-OH[61] coupled in the presence of DCC (3 equiv. of each reagent, 2–4 h). Hydroxyl groups were esterified by reaction with the C-terminal Boc-amino acid and DCC (10 equiv.) in the presence of 0.5 equiv. of DMAP for 2 h. The coupling yield was determined from the relative proportion [C-terminal residue]/[internal reference amino acid] after acid hydrolysis and amino acid analysis (typical values: 0.93–0.97, 0.79 in the case of **9**), and unreacted hydroxyl groups were capped by reaction with benzoyl chloride (or acetic anhydride) and pyridine.

The remaining amino acids of the peptide sequence were incorporated following the standard procedures for solid-phase peptide assembly (3 equiv. of Boc-amino acid and DCC and 1–1.5 h reaction time were used for the coupling step), with the addition of an equimolar amount of HOBt in the incorporation of the homoserine[59] and $Arg(Fmoc)_2$ derivatives. When arginine was protected by protonation, a two-fold excess of HOBt was added during the coupling step, and, after the incorporation of this residue, the TFA-mediated removal of the Boc group was followed by neutralization with a 5% DIEA solution in DCM and treatment with a 0.5 M solution of HOBt in DMF (4×1 min). The N^{α}-Boc derivative of the linking amino acid was incorporated with the hydroxyl group unprotected. Acylation of the N-terminal amine group was carried out by reaction with acetic anhydride. The homoserine derivative Boc-Hse(DMT)-O^- $HTEA^+$ was prepared as previously described.[59]

Oligonucleotide elongation was carried out (5–10 μmol-scale) following standard phosphite triester procedures with the minor modifications that render this methodology compatible with the use of polystyrene supports.[60] 0.15–0.2 M solutions of commercially available 3′-phosphoramidite derivatives (5′-DMT-N-P(OCNE)$NiPr_2$, N=T, A^{Bz}, C^{Bz}, G^{iBu}) in anhydrous DCM and 0.7–0.8 M solutions of tetrazole in anhydrous THF were used for the coupling step (30 min coupling time for the first nucleoside and 15 min for the others). 1 M *t*-BuOOH was always used for the oxidation of phosphites to phosphates during the oligonucleotide elongation. The 5′-DMT group was removed from the nucleopeptide-resin. Nucleopeptides were detached from the solid support by treatment with a 1:1 conc. aq. ammonia/dioxane solution (dioxane was first added to swell the polystyrene resin). Overall synthesis, deprotection and purification yields ranged between the following values: nucleopeptides **13** and **14**: 25–30%; nucleopeptides **1** and **2**: 15–20%; nucleopeptides **3**, **4**, **11** and **12**: 10–15%; nucleopeptides **5**, **6**, **9** and **10**: 1–5%.

4.2. Ac-Tyr(p$^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH (1)

Peptide-resins: Ac-Tyr-Leu-Asp(OFm)-Pro-Arg(X)-Ile-Thr(Ac)-Val-*O*-HMFS-Phe-MBHA-PS (X=H/$Fmoc_2$, 0.14–0.15 mmol/g). Amino acid analysis after acid hydrolysis: X=H: Asp 0.97, Thr 0.36, Val 1.66, Ile 0.78, Leu 1.01, Tyr 1.00, Arg 1.01, Pro 1.00; X=$Fmoc_2$: Asp 1.02, Thr 0.39, Val 1.68, Ile 0.84, Leu 1.03, Tyr 1.02, Arg 1.08, Pro 0.86. Crude nucleopeptide was obtained by overnight ammonia treatment of the two nucleopeptide-resins at 55°C. The product obtained from X=$Fmoc_2$ was purified by semi-preparative reversed-phase HPLC (10–40% of B in 30 min).

Amino acid composition after acid hydrolysis: Asp 0.41, Thr 0.44, Pro 1.28, Val 1.17, Ile 0.82, Leu 1.33, Tyr 1.47, Arg 1.09. Nucleoside composition after enzymatic digestion: dC/dG/T/dA: 2.12/2.05/4.95/5.92. Compound **1**: $C_{195}H_{260}N_{67}O_{103}P_{15}$, calcd mass: 5655.19 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, linear), *m/z*: 5651.6 $[M-H]^-$; ES MS (negative mode), *m/z*: 1412.5 $[M-4H]^{4-}$, 1130.2 $[M-5H]^{5-}$, 941.6 $[M-6H]^{6-}$, 807.2 $[M-7H]^{7-}$, mass found: 5652.

4.3. Ac-Tyr(p$^{3'}$dTTTCAGAA)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH (2)

Peptide-resins: the same as above. Ammonia final deprotection treatment: overnight, 55°C. The product obtained from X=$Fmoc_2$ was purified by semi-preparative reversed-phase HPLC (10–40% of B in 30 min).

Amino acid composition after acid hydrolysis: Asp 0.57, Thr 0.66, Pro 1.83, Val 1.21, Ile 0.71, Leu 0.80, Tyr 1.31, Arg 0.91. Nucleoside composition after enzymatic digestion: dC/dG/T/dA: 1.06/1.02/2.91/3.05. Compound **2**: $C_{126}H_{174}N_{40}O_{62}P_8$, calcd mass: 3488.78 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, linear), *m/z*: 3487.0 $[M-H]^-$; ES MS (negative mode), *m/z*: 1743.1 $[M-2H]^{2-}$, 1162.0 $[M-3H]^{3-}$, 871.3 $[M-4H]^{4-}$, 697.0 $[M-5H]^{5-}$, mass found: 3487.

4.4. Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-OH (3) and Ac-Asn-Phe-Val-Ser(p$^{3'}$TTTT)-Gly-NH_2 (4)

Peptide-resin: Ac-Asn-Phe-Val-Ser-Gly-*O*-HMFS-Leu-MBHA-resin (0.21 mmol/g). Amino acid analysis after acid hydrolysis: Asp 1.11, Ser 0.47, Gly 1.01, Val 1.03, Phe 0.86. Ammonia final deprotection treatment: 6 h, rt. Purification: MPLC (glass column, 22×2 cm, Vydac C-18), gradient from 0 to 30% of B, 600 mL of each solvent. Isolated products: TpTpTpTp ($C_{40}H_{54}N_8O_{29}P_4$, calcd mass: 1234.8 (average), 1234.2 (monoisotopic): MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron),

m/z: 1233.5 $[M-H]^-$. Ac-Asn-Phe-Val-Ser(p$^{3'}$dTTTT)-Gly-OH (**3**, $C_{65}H_{88}N_{14}O_{37}P_4$, calcd mass: 1781.4 (average)): MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron), *m/z*: 1779.2 $[M-H]^-$. Ac-Asn-Phe-Val-Ser(p$^{3'}$dTTTT)-Gly-NH_2 (**4**, $C_{65}H_{89}N_{15}O_{36}P_4$, calcd mass: 1780.4 (average)): MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron), *m/z*: 1778.1 $[M-H]^-$.

4.5. H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH (5)

Peptide-resin: Fmoc-Ile-Ala-Leu-Gly-Thr(Ac)-Ser(Ac)-Lys(Tfa)-Leu-Asn-Tyr-Leu-Asp(OFm)-Pro-*O*-HMFS-Phe-MBHA-resin (0.13 mmol/g). To prevent diketopiperazine formation from H-Asp-Pro-resin, the Boc group of Boc-Asp(OFm)-Pro-resin was removed as usual, and Boc-leucine (5 equiv.) was coupled to the dipeptide-resin (trifluoroacetate salt) by reaction with PyAOP (7-azabenzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) and DIEA (10 equiv.). The N-terminal amino acid was incorporated as Fmoc-derivative. Amino acid analysis after acid hydrolysis: Asp 2.15, Thr 0.65, Ser 0.33, Gly 0.87, Ala 0.95, Ile 0.60, Leu 1.97, Tyr 1.09, Lys 1.02, Pro 1.04. HPLC analysis of crude nucleopeptide after overnight ammonia deprotection at 55°C (10–40% of B in 30 min): t_R=11.7 (≈20%, impurity 1, **7**), 24.5 (≈30%, **5**) and 25.5 min (≈30%, impurity 2, **8**). Purification: HPLC (10–40% of B in 30 min).

Compound **5**: calcd mass for $C_{103}H_{154}N_{30}O_{44}P_4$: 2640.4 (average). Amino acid composition after enzymatic digestion: Asp 0.88, Thr 0.73, Ser 1.05, Asn 0.79, Pro 1.72, Gly 1.75, Ala 1.51, Ile 1.11, Leu 1.52, Tyr 1.09, Lys 0.90. Nucleoside composition after enzymatic digestion: dC/dG/T/dA: 1.25/1.19/1.06/0.51. MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron): *m/z*: 2639.1 $[M-H]^-$.

Compound **7**: H-Tyr(p$^{3'}$dACGT)-Leu-Asp-Pro-OH. Calcd mass $C_{63}H_{83}N_{19}O_{32}P_4$: 1742.4 (average). MALDI-TOF MS (negative mode, reflectron), *m/z*: 1740.6 $[M-H]^-$.

Compound **8**: MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron), *m/z*: 2639.2 $[M-H]^-$.

4.6. H-Ile-Ala-Leu-Gly-Thr-Ser-Lys-Leu-Asn-Tyr(p$^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-OH (6)

Peptide-resin: the same as above. Final ammonia deprotection: overnight, 55°C. Purification: preparative PAGE, followed by HPLC separation (10–40% of B in 30 min) of the two products contained in the main electrophoretic band (t_R=13.6 and 14.2 min).

Compound **6**: calcd mass for $C_{212}H_{290}N_{71}O_{109}P_{15}$: 6041.6 (average). Amino acid analysis after enzymatic digestion: Asp 0.65, Thr 0.71, Ser 1.09, Asn 1.13, Pro 1.92, Gly 2.00, Ala 1.36, Ile 0.78, Leu 1.06, Tyr 1.45, Lys 0.85. Nucleoside composition after enzymatic digestion: dC/dG/T/dA: 3.05/2.04/4.23/5.77. MALDI-TOF MS (2,4,6-THAP, negative mode): *m/z*: 6036.3 $[M-H]^-$. Impurity: MALDI-TOF MS (2,4,6-THAP, negative mode, linear): *m/z*: 6036.9.

4.7. Ac-Cys-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH (9)

Cysteine derivative used: Ac-Cys(*S*–*t*Bu)-OH. Peptide-resin: Ac-Cys(*S*–*t*Bu)-Gly-Tyr-Pro-*O*-HMFS-Phe-MBHA-resin (0.19 mmol/g). Final deprotection conditions: conc. aq. ammonia/[1 M DTT in dioxane] 1:1, 7 h, 55°C. Purification: (i) gel filtration (Sephadex G-15, 0.05 M ammonium acetate) to remove the excess of DTT; (ii) semi-preparative HPLC on a Hamilton PRP-1 column (305×7 mm, 10 μm, 1.5 mL/min), A=0.01 M ammonium acetate, B=acetonitrile/H_2O 1:1, linear gradient from 5 to 35% of B in 30 min. The two main products isolated (t_R=16.2 and 17.3 min) were the two conformational isomers of the Tyr-Pro peptide bond in **9**. A third minor product was also isolated (t_R=19.5 min). Treatment of **9** with a 10% aqueous DMSO solution afforded, almost quantitatively, the product eluting at 19.5 min, which was characterized as the disulfide dimer form of **9**. This product was purified using the same conditions as for the nucleopeptide monomer.

Compound **9**: calcd mass for $C_{80}H_{102}N_{26}O_{43}P_6S$: 2333.7 (average). Nucleoside composition after enzymatic digestion: dC/dG7T7dA: 1.31/1.10/2.10/1.49. MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron): *m/z* 2332.3 $[M-H]^-$; electrospray MS: *m/z* 776.9 $[M-3H]^{3-}$, 1165.3 $[M-2H]^{2-}$, calcd mass: 2333.24±0.58.

4.8. {Ac-Cys-Gly-Tyr(p$^{3'}$dACTAGT)-Pro-OH}$_2$ [S–S]

Disulfide dimer of **9**: calcd mass for $C_{160}H_{202}N_{52}O_{86}P_{12}S_2$: 4665.4 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, linear): *m/z* 4670.5 $[M-H]^-$; electrospray MS: *m/z* 663.9 $[M-7H]^{7-}$, 776.7 $[M-6H]^{6-}$, 1166.1 $[M-4H]^{4-}$, calcd mass: 4663.8±5.4.

4.9. Ac-Cys-Gly-Ala-Hse(p$^{3'}$dGCATGC)-Ala-OH (10)

Cysteine derivative used: Boc-Cys(*S*–*t*Bu)-OH. The N-terminal was acetylated by reaction with AcOH (5 equiv.) and DCC (5 equiv.) for 30 min. Peptide-resin: Ac-Cys(*S*–*t*Bu)-Gly-Ala-Hse-Ala-*O*-HMFS-Leu-MBHA-resin (0.20 mmol/g). The oligonucleotide chain was assembled using the standard 3′-phosphoramidite derivatives of T, A^{Bz}, C^{Ac} and G^{Dmf} (Glen Res. products, Eurogentec, BE). Deprotection and cleavage: treatment with conc. aq. ammonia/[1 M DTT in dioxane] 1:1, 6 h, rt. DTT was removed by gel filtration (Sephadex G-15, 0.05 M ammonium acetate) and pure **10** was isolated after MPLC purification (glass column, 22×2 cm, Vydac C-18, gradient from 0 to 30% of B, 600 mL of each solvent). **10** was dissolved (ca. 50 μM) in 60 mM triethylammonium acetate buffer, pH=8, and air-oxidized by vigorous magnetic stirring for 2 days at rt. The disulfide dimer of **10** was purified by semi-preparative HPLC (5–25% of B in 40 min) followed by several runs of analytical HPLC (5–25% of B in 40 min, analytical Kromasil column).

Compound **10**: calcd mass for $C_{75}H_{102}N_{28}O_{44}P_6S$: 2317.7 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron): *m/z* 2315.5 $[M-H]^-$.

4.10. Ac-Cys(*S*–*t*Bu)-Gly-Ala-Hse-Ala-OH

Obtained from cleavage of an aliquot of peptide-resin with conc. aq. ammonia/dioxane 1:1, 4 h, rt): calcd mass for $C_{21}H_{37}N_5O_8S_2$: 551.7 (average). MALDI-TOF MS (2,5-dihydroxybenzoic acid, negative mode, reflectron): *m/z* 550.6 $[M-H]^-$.

4.11. Ac-Cys-Gly-Ala-Hse-Ala-OH

Obtained from cleavage of an aliquot of peptide-resin with conc. aq. ammonia/[1 M DTT in dioxane] 1:1, 5 h, rt, and removal of DTT by extraction with ethyl acetate): calcd mass for $C_{17}H_{29}N_5O_8S$: 463.5 (average). MALDI-TOF MS (2,5-dihydroxybenzoic acid, positive mode, reflectron): *m/z* 465.2 $[M+H]^+$, 487.3 $[M+Na]^+$, 503.3 $[M+K]^+$.

4.12. {Ac-Cys-Gly-Ala-Hse(p$^{3'}$dGCATGC)-Ala-OH}$_2$ [S–S]

Disulfide dimer of **10**: calcd mass for $C_{150}H_{202}N_{56}O_{88}P_{12}S_2$: 4633.4 (average). Nucleoside composition after enzymatic digestion: dC 2.2, dG 0.7, T 2.2, dA 1.0.MALDI-TOF MS (2,4,6-THAP, negative mode, linear): *m/z* 4628.9 $[M-H]^-$; 2316.5 $[M-2H]^{2-}$.

4.13. Ac-Gly-Ser(p$^{3'}$T$_5$)-Leu-Ala-Val-OH, 11, and Ac-Gly-Thr(p$^{3'}$T$_5$)-Leu-Ala-Val-OH (12)

Peptide-resins: Ac-Gly-Xxx-Leu-Ala-Val-*O*-HMFS-Phe-resin, Xxx=Ser, Thr (0.19 mmol/g). Final ammonia deprotection treatment: 2 h, rt. Purification: anal HPLC, linear gradient from 5 to 35% of B in 30 min.

Compound **11**: calcd mass for $C_{71}H_{102}N_{15}O_{43}P_5$ 2008.53 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron) *m/z* 2006.50 $[M-H]^-$.

Compound **12**: calcd mass for $C_{72}H_{104}N_{15}O_{43}P_5$ 2022.56 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron) *m/z* 2020.50 $[M-H]^-$.

4.14. Ac-Gly-Tyr(p$^{3'}$T$_5$)-Leu-Ala-Val-OH (13), and Ac-Gly-Tyr(p$^{5'}$T$_5$)-Leu-Ala-Gly-OH (14)

Peptide-resin: Ac-Gly-Tyr-Leu-Ala-Val-*O*-HMFS-Phe-resin (0.19 mmol/g). Final ammonia deprotection treatment: 6 h, rt. Purification: anal HPLC, linear gradient from 5 to 35% of B in 30 min.

Compound **13**: calcd mass for $C_{77}H_{106}N_{15}O_{43}P_5$: 2084.63 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron) *m/z* 2082.48 $[M-H]^-$.

Compound **14**: calcd mass for $C_{77}H_{106}N_{15}O_{43}P_5$: 2084.63 (average). MALDI-TOF MS (2,4,6-THAP, negative mode, reflectron) *m/z* 2082.65 $[M-H]^-$.

4.15. Ac-Cys(*S*–*t*Bu)-OH

H-Cys(*S*–*t*Bu)-OH (Novabiochem, 3 g, 14.3 mmol) was treated with a two-fold molar excess of acetic anhydride and *N*-ethyl-*N*,*N*-diisopropylamine for 1 h at rt. The organic phase obtained after extraction of the reaction mixture with 0.1 M HCl (2×) and that obtained from reextraction of the aqueous phase with methylene chloride was dried over sodium sulfate, filtered and evaporated to dryness. Pure Ac-Cys(*S*–*t*Bu)-OH was obtained after recrystallization from hexanes/dichloromethane (76% yield): mp 176–177°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.9 (s, 9H), 2.1 (s, 3H), 3.1 (m, CH_2), 3.7 (m, CH). Calcd mass for $C_9H_{17}NO_3S_2$: 251.4 (average), CI MS (NH_3, positive mode): *m/z* 269 $[M+18]^+$, 286 $[M+35]^+$.

4.16. Boc-Cys(*S*–*t*Bu)-OH

H-Cys(*S*–*t*Bu)-OH (Novabiochem, 0.5 g, 2.4 mmol) was suspended in 15 mL of a 2:1 dioxane/H_2O mixture, and the pH was adjusted to 9 by addition of 1 M NaOH. After addition of 0.60 g of di-*tert*-butyldicarbonate (Boc_2O, 2.74 mmol), the reaction was kept overnight at rt (magnetic stirring). After extraction with hexanes (2×50 mL), the aqueous phase was chilled and acidified to pH 2–3 by addition of 1 M HCl. The mixture was extracted with ethyl acetate (3×50 mL), and the combined organic phases were washed with H_2O (2×25 mL), dried over sodium sulfate, filtered and evaporated to dryness. Pure Boc-Cys(*S*–*t*Bu)-OH was obtained (80% yield) without further purification: mp 119–120°C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 5.4 (d, 1H), 4.6 (m, 1H), 3.2 (m, 2H), 1.5 (s, 3H), 1.3 (s, 3H). Calcd mass for $C_{12}H_{23}NO_4S_2$: 309.5 (average), FAB MS (4-nitrobenzyl alcohol, positive mode): *m/z* 309.4 $[M+H]^+$, 332.4 $[M+Na]^+$.

Acknowledgments

Laurent Debéthune was a recipient fellow of the Generalitat de Catalunya, and Vicente Marchán of the Universitat de Barcelona. This work was supported by funds from the Spanish MEC (grants PB97-0941-C02-01 and BQU 3693-C02-01) and the Generalitat de Catalunya (2000SGR18, 2001SGR49 and Centre de Referència de Biotecnologia).

References

1. Salas, M. *Annu. Rev. Biochem.* **1991**, *60*, 39–71.
2. Wang, J. C. *Annu. Rev. Biochem.* **1996**, *65*, 635–692.
3. Tung, C-H.; Stein, S. *Bioconjugate Chem.* **2000**, *11*, 605–618, and references therein.
4. Tong, G.; Lawlor, J. M.; Tregear, G. W.; Haralambidis, J. *J. Org. Chem.* **1993**, *58*, 2223–2231.
5. Debéthune, L.; Kohlhagen, G.; Grandas, A.; Pommier, Y. *Nucleic Acids Res.* **2002**, *30*, 1198–1204.
6. Civitello, E. R.; Leniek, R. G.; Hossler, K. A.; Haebe, K.; Stearns, D. M. *Bioconjugate Chem.* **2001**, *12*, 459–463.
7. Beltrán, M.; Onoa, G. B.; Pedroso, E.; Moreno, V.; Grandas, A. *J. Biol. Inorg. Chem.* **1999**, *4*, 701–707.
8. Marchán, V.; Moreno, V.; Pedroso, E.; Grandas, A. *Chem. Eur. J.* **2001**, *7*, 808–815.
9. Stetsenko, D. A.; Gait, M. J. *Bioconjugate Chem.* **2001**, *12*, 576–586.
10. Robles, J.; Maseda, M.; Beltrán, M.; Concernau, M.; Pedroso, E.; Grandas, A. *Bioconjugate Chem.* **1997**, *8*, 785–788.

11. Robles, J.; Pedroso, E.; Grandas, A. *Nucleic Acids Res.* **1995**, *23*, 4151–4161.
12. Dreef-Tromp, C. M.; van Dam, E. M. A.; van den Elst, H.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1990**, *18*, 6491–6495.
13. Dreef-Tromp, C. M.; van den Elst, H.; van der Boogart, J. E.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1992**, *20*, 2435–2439.
14. Dreef-Tromp, C. M.; van der Maarel, J. C. M.; van den Elst, H.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1992**, *20*, 4015–4020.
15. Ueno, Y.; Saito, R.; Hata, T. *Nucleic Acids Res.* **1993**, *21*, 4451–4457.
16. Waldmann, H.; Gabold, S. *Chem. Commun*. **1997**, 1861–1862.
17. Flohr, S.; Jungmann, V.; Waldmann, H. *Chem. Eur. J.* **1999**, *5*, 669–681.
18. Sakakura, A.; Hayakawa, Y.; Harada, H.; Hirose, M.; Noyori, R. *Tetrahedron Lett.* **1999**, *40*, 4359–4362.
19. Sakakura, A.; Hayakawa, Y. *Tetrahedron* **2000**, *56*, 4427–4438.
20. Sakakura, A.; Hayakawa, A. *Tetrahedron* **2000**, *56*, 4427–4435.
21. McMinn, D. L.; Matray, T. J.; Greenberg, M. M. *J. Org. Chem.* **1997**, *62*, 7074–7075.
22. Kahl, J. D.; McMinn, D. L.; Greenberg, M. M. *J. Org. Chem.* **1998**, *63*, 4870–4871.
23. Kahl, J. D.; Greenberg, M. M. *J. Org. Chem.* **1999**, *64*, 507–510.
24. Marchán, V.; Rodríguez-Tanty, C.; Estrada, M.; Pedroso, E.; Grandas, A. *Eur. J. Org. Chem.* **2000**, 2495–2500.
25. Beltrán, M.; Pedroso, E.; Grandas, A. *Tetrahedron Lett.* **1998**, *39*, 4115–4118.
26. Robles, J.; Beltrán, M.; Marchán, V.; Pérez, Y.; Travesset, I.; Pedroso, E.; Grandas, A. *Tetrahedron* **1999**, *55*, 13251–13264.
27. Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* **2000**, *41*, 9113–9117.
28. Camble, R.; Petter, N. N. Fragment Condensation and Protonated Arginine in Solid Phase Synthetic Studies on Sequence 1–47 of Urogastrone. In *Peptides 1976. Proceedings of the Fourteenth European Peptide Symposium*; Loffet, A., Ed.; Éditions de l'Université de Bruxelles: Bruxelles, 1976; pp 299–307.
29. Farrow, M. A.; Aboul-ela, F.; Owen, D.; Karpeisky, A.; Beigelman, L.; Gait, M. J. *Biochemistry* **1998**, *37*, 3096–3108.
30. Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *Special Methods in Peptide Synthesis, Part A. The Peptides. Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1980; Vol. 2, p 174.
31. Robles, J.; Pedroso, E.; Grandas, A. *J. Org. Chem.* **1995**, *60*, 4856–4861.
32. Filippov, D.; Meeuwenoord, N. J.; van der Marel, G. A.; Efimov, V. A.; Kuyl-Yeheskiely, E.; van Boom, J. A. *Synlett* **1996**, 769–771.
33. Moriguchi, T.; Yanagi, T.; Kunimori, M.; Wada, T.; Sekine, M. *J. Org. Chem.* **2000**, *65*, 8229–8238.
34. Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *Special Methods in Peptide Synthesis, Part A. The Peptides. Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1980; Vol. 2, pp 193–196.
35. Yang, Y.; Sweeney, W. V.; Schneider, K.; Thörnqvist, S.; Chait, B. T.; Tam, J. P. *Tetrahedron Lett.* **1994**, *35*, 9689–9692.
36. Schirmeister, H.; Himmelsbach, F.; Pfleiderer, W. *Helv. Chim. Acta* **1993**, *76*, 385–401.
37. Yoshifuji, S.; Tanaka, K.-i.; Nitta, Y. *Chem. Pharm. Bull.* **1985**, *33*, 1749–1751.
38. Tanaka, K-i.; Yoshifuji, S.; Nitta, Y. *Chem. Pharm. Bull.* **1988**, *36*, 3125–3129.
39. García de la Torre, B.; Albericio, F.; Saison-Behmoaras, E.; Bachi, A.; Eritja, R. *Bioconjugate Chem.* **1999**, *10*, 1005–1012.
40. Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *Special Methods in Peptide Synthesis, Part A. The Peptides. Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1980; Vol. 2, p 204.
41. Barany, G.; Merrifield, R. B. Solid-Phase Peptide Synthesis. In *Special Methods in Peptide Synthesis, Part A. The Peptides. Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1980; Vol. 2, pp 206–207.
42. Soukchareun, S.; Haralambidis, J.; Tregear, G. *Bioconjugate Chem.* **1998**, *9*, 466–475.
43. Schwope, I.; Bleczinski, C. F.; Richert, C. *J. Org. Chem.* **1999**, *64*, 4749–4761.
44. Gottschling, D.; Seliger, H.; Tarrasón, G.; Piulats, J.; Eritja, R. *Bioconjugate Chem.* **1998**, *9*, 831–837.
45. Tam, J. P.; Wu, C.-R.; Liu, W.; Zhang, J.-W. *J. Am. Chem. Soc.* **1991**, *113*, 6657–6662.
46. Wilk, A.; Grajkowski, A.; Srinivasachar, K.; Beaucage, S. L. *Antisense Nucleic Acid Drug Dev.* **1999**, *9*, 361–366.
47. Shabarova, Z. A In *Synthetic Nucleotide-Peptides*. *Progress in Nucleic Acid Research and Molecular Biology*; Davidson, J. N., Colum, W. E., Eds.; Academic: London, 1970; Vol. 10, pp 145–182.
48. Beltrán, M. PhD Thesis, University of Barcelona, 1999.
49. Antopolski, M.; Azhayev, A. *Helv. Chim. Acta* **1999**, *82*, 2130–2140.
50. Kuyl-Yeheskiely, E.; Tromp, C. M.; Lefeber, A. W. M.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron* **1988**, *44*, 6515–6523.
51. Kuyl-Yeheskiely, E.; Dreef-Tromp, C. M.; Geluk, A.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1989**, *17*, 2897–2905.
52. Estrada, M.; Robles, J.; Grandas, A. Unpublished results.
53. Marchán, V. PhD Thesis, University of Barcelona, 2000.
54. Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett.* **1994**, *35*, 4311–4314.
55. Schulhof, J. C.; Molko, D.; Teoule, R. *Tetrahedron Lett.* **1987**, *28*, 51–54.
56. McBride, L. J.; Kierzek, R.; Beaucage, S. L.; Caruthers, M. H. *J. Am. Chem. Soc.* **1986**, *108*, 2040–2048.
57. Sinha, N. D.; Davis, P.; Usman, N.; Pérez, J.; Hodge, R.; Kremsky, J.; Casale, R. *Biochimie* **1993**, *75*, 13–23.
58. Stetsenko, D. A.; Gait, M. J. *J. Org. Chem.* **2000**, *65*, 4900–4908.
59. Beltrán, M.; Maseda, M.; Robles, J.; Pedroso, E.; Grandas, A. *Lett. Pept. Sci.* **1997**, *4*, 147–155.
60. Montserrat, F. X.; Grandas, A.; Eritja, R.; Pedroso, E. *Tetrahedron* **1994**, *50*, 2617–2622.
61. Rabanal, F.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **1992**, *33*, 1775–1778.