Stepwise Solid-Phase Synthesis of Nucleopeptides

Modification of oligonucleotides by attachment of peptide chains has attracted considerable interest during the last few years. The covalent union of a peptide chain may have beneficial effects on synthetic oligonucleotides (Tung and Stein, 2000), such as (1) increasing their stability to exonucleases, (2) accelerating or enhancing hybridization to their target sequences, and (3) facilitating their transport through cell membranes, which is particularly important for antisense (or antigene) applications (Gait, 2003).

No rules are established to name and describe the different types of peptide-oligonucleotide conjugates. The word nucleoprotein is used to describe both covalently and noncovalently linked nucleic acid–protein complexes. Since in the former a phosphate group links the 5′- or 3′-terminal hydroxyl group of a nucleic acid with the side-chain hydroxyl group of serine, threonine, or tyrosine of a protein, phosphodiester-linked peptide-oligonucleotide conjugates have often been denoted as nucleopeptides (Schattenkerk et al., 1984; Robles et al., 1991; Waldmann and Gabold, 1997). Some authors, however, have used the term nucleopeptide to refer to other types of conjugates (*UNIT 4.5*; McMinn and Greenberg, 1999), and still others have referred to nucleopeptides as peptide-oligonucleotide conjugates (see below; Stetsenko et al., 2002).

This unit describes stepwise solid-phase methods to obtain nucleopeptides containing 2′-deoxynucleosides and any of the proteinogenic amino acids, including the trifunctional ones. The procedures reported here are also suitable for the preparation of other peptide-oligonucleotide conjugates, since most of the critical points of the procedures are related to the presence of purine nucleosides and some trifunctional amino acids in the target molecule. Methods for synthesis of the desired support-bound peptide (see Basic Protocol 1) are followed by methods for preparing the solid support (see Support Protocol 1) and several protected amino acids that are not commercially available (see Support Protocols 2 to 5). This is followed by extension of the oligonucleotide and cleavage and deprotection of the nucleopeptide (see Basic Protocol 2), and analysis, purification, and characterization of the target molecule (see Basic Protocols 3 and 4). Additional methods are given for reduction of nucleopeptides containing sulfoxide-protected methionine (see Support Protocol 6) and deprotection and purification of cysteine-containing nucleopeptides (see Support Protocol 7).

STRATEGIC PLANNING

Synthesis Strategy

The synthesis of any type of peptide-oligonucleotide conjugate is a chemical challenge, because two completely different chemical entities must be assembled in the same molecule. Since the beginning of this work, the aim of the authors was to develop the simplest possible methodology for the preparation of nucleopeptides. In this respect, it was clear that the use of solid-phase chemistry would be one of the keys to simplifying the overall procedure.

Both peptides and oligonucleotides are usually obtained by well-established solid-phase procedures, but the two methodologies differ considerably in many aspects. First, the coupling step is obviously different. In peptide synthesis, carboxylic acids have to be activated to form amide bonds with the free amine groups immobilized on the solid support, while elongation of oligonucleotide chains requires a phosphoric ester to be formed between two hydroxyl groups. Second, the protecting groups required at every

Contributed by Anna Grandas, Vicente Marchán, Laurent Debéthune and Enrique Pedroso
Current Protocols in Nucleic Acid Chemistry (2004) 4.22.1-4.22.54
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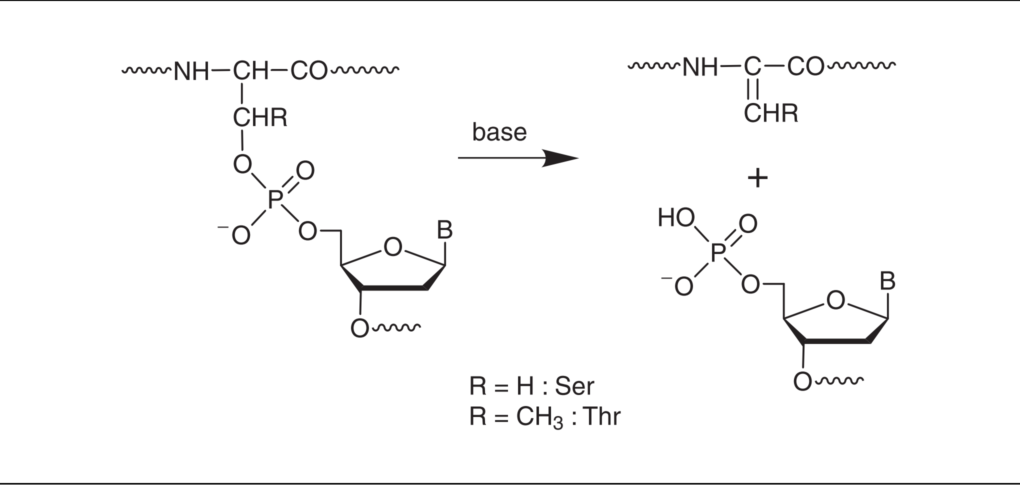

Figure 4.22.1 Base-promoted cleavage of nucleopeptides with serine- or threonine-nucleoside phosphodiester bonds.

step to mask the appropriate functionalities—and, consequently, the treatments necessary for their removal—are also quite different.

Peptide chains are commonly obtained either by using groups that are labile to acids of different strength (the so-called Boc/benzyl strategy) or by using a combination of base-labile temporary α-amine-protecting groups and acid-labile permanent side-chain-protecting groups (Fmoc/*t*Bu strategy). As most readers know, oligonucleotide synthesis combines the use of very mild acidic conditions to eliminate the temporary protecting groups (in order to prevent depurination) with that of a basic ammonia treatment to remove the permanent protecting groups (see *UNIT 2.1*). This scenario illustrates that the two methodologies cannot easily be made compatible, as the standard final treatment in peptide synthesis is a reaction in acidic conditions, under which an oligonucleotide chain would not survive. Another point of concern is the stability of the [hydroxylated amino acid]-nucleoside phosphodiester linkage, which can undergo a β-elimination process that degrades the target molecule (Shabarova, 1970). Figure 4.22.1 shows the β-elimination side reaction that may affect nucleopeptides (depurination is shown in Figure 2.1.8).

The synthesis protocol described here is a stepwise approach (Fig. 4.22.2). It has been optimized so that the methodology is accessible to groups of nonspecialists in the field and, whenever possible, is compatible with commercially available synthons.

Taking into account that peptide chains are normally elongated from the C to N terminus and oligonucleotides from the 3′ to 5′ terminus (although reverse elongation is also possible), elongation of the oligonucleotide chain at the side-chain hydroxyl of a resin-linked peptide seemed the best alternative. First, this allows greater stability, as the peptide is more robust than the oligonucleotide, particularly to acidic treatments. Second, this allows the linking amino acid to be placed at any position in the peptide chain. Stepwise assembly of the peptide moiety on a resin-linked oligonucleotide would only yield nucleopeptides with the linking amino acid at the C terminus. Moreover, difficulties in coupling amino acids onto resin-linked oligonucleotides have in some cases been reported (Bergmann and Bannwarth, 1995; Marchán et al., 2000).

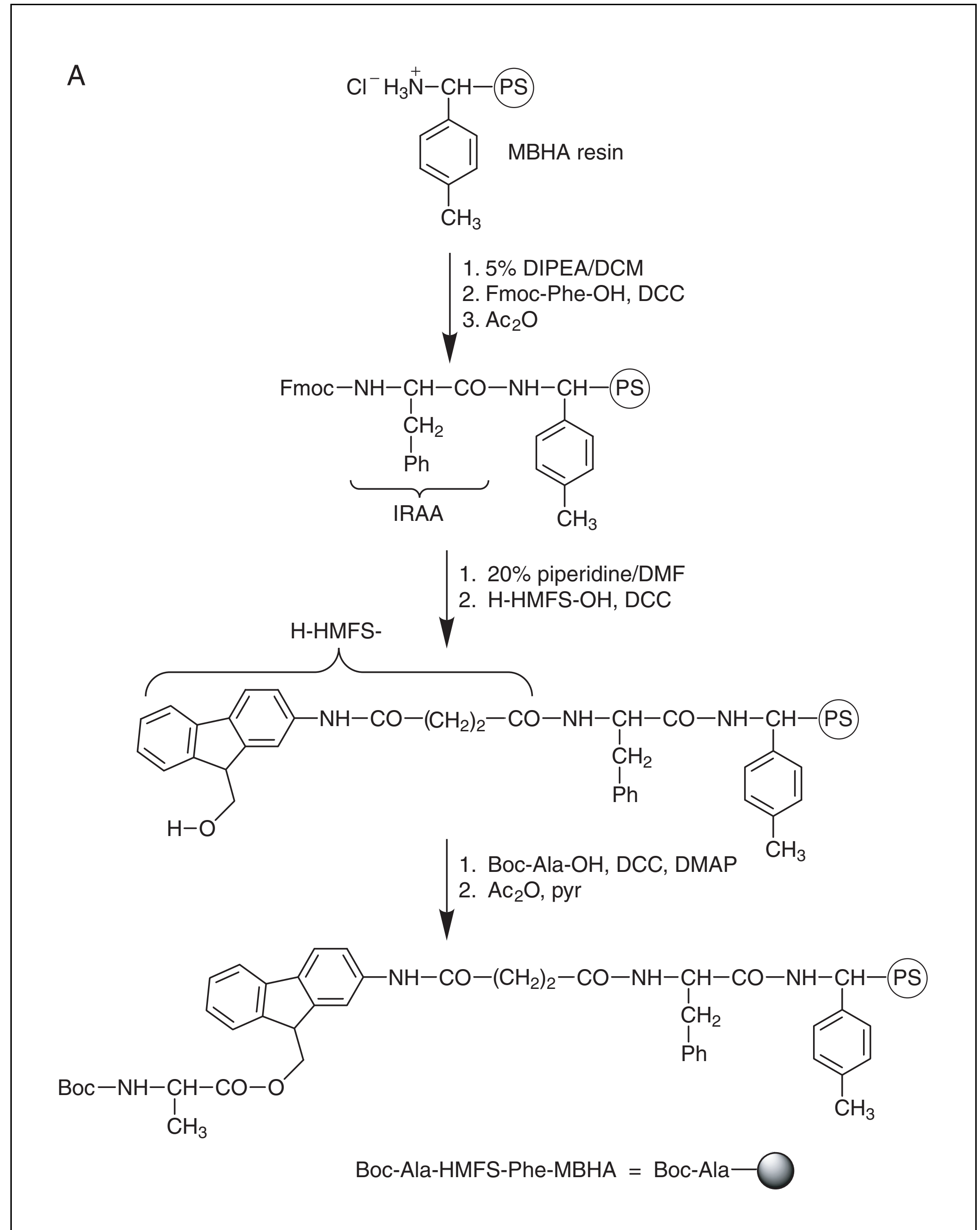

Figure 4.22.2 *(continues on next page)* Stepwise solid-phase synthesis of nucleopeptide Ac-Lys-Trp-Lys-Hse(p3édGCATCG)-Ala-OH. (A) Preparation of the [C-terminal amino acid]-resin includes coupling of an IRAA and an HMFS linker (handle) to the resin (see Support Protocol 1), followed by coupling of the C-terminal amino acid (see Basic Protocol 1). (**B**) Peptide assembly (see Basic Protocol 1) followed by oligonucleotide assembly and final deprotection (see Basic Protocol 2) yield the crude target nucleopeptide. [1]HOBt must be added to couple homoserine. Bases: B_1 and B_6, N^2-isobutyrylguanin-9-yl; B_2 and B_5, N^4-benzoylcytosin-1-yl; B_3, N^6-benzoyladenin-9-yl; B_4, thymin-1-yl. Other abbreviations: aa, amino acid; Boc, *tert*-butoxycarbonyl; CNE, 2-cyanoethyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-(*N,N*-dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; DMTr, 4,4′-dimethoxytrityl; dN, 2′-deoxyribonucleoside; Fmoc, 9-fluorenylmethoxycarbonyl; For, formyl; HOBt, 1-hydroxybenzotriazole; Hse, homoserine; H-HMFS-OH, *N*-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid; IRAA, internal reference amino acid; MBHA, *p*-methylbenzhydrylamine; NMI, *N*-methylimidazole; PS, polystyrene-*co*-1%-divinylbenzene; pyr, pyridine; TCA, trichloroacetic acid; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid.

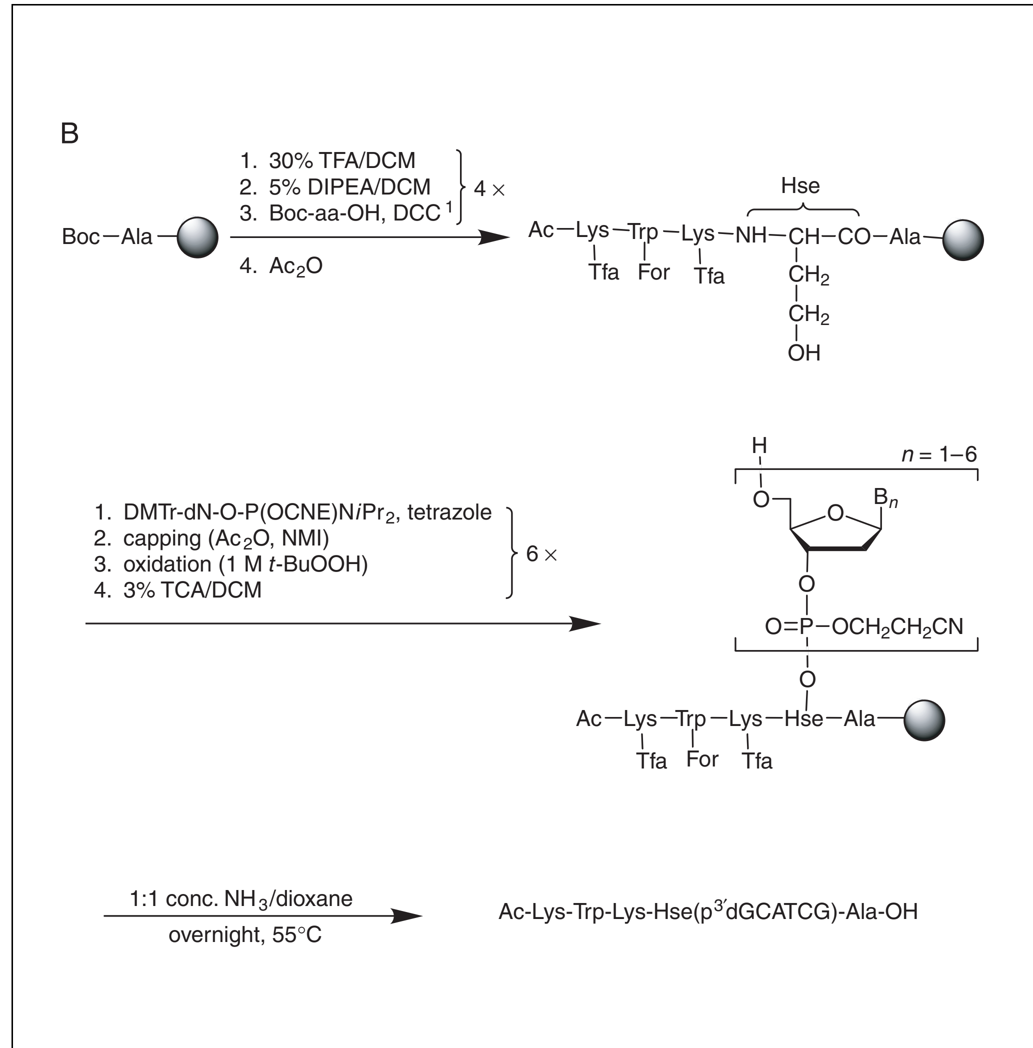

Figure 4.22.2 *continued.*

Oligonucleotide assembly onto a resin-linked peptide is usually carried out with 3′-phosphoramidite nucleoside derivatives, which links the peptide to the 3′ end of the oligonucleotide. Substitution of 5′-phosphoramidites for the 3′-derivatives allows peptide-5′-oligonucleotides to be obtained.

Choice of Protecting Groups

The main concern in the overall process is the choice of protecting groups. They must be chosen so that the integrity of the target molecule is not affected by the final deprotection conditions. This means, as previously stated, avoiding the use of harsh acidic and basic treatments. Peptide assembly is compatible with the use of N^{α}-Boc-protected amino acids, and, although they are often commercially available with acid-labile side-chain-protecting groups, some derivatives with base-labile groups at the side chain are available. The only problem, then, is the peptide-oligonucleotide phosphate union, which in some cases can be particularly labile to bases. Since the data available at the outset of this work suggested that basic treatments could to some extent (though not completely) degrade nucleopeptides in which serine or threonine are the linking amino acids, it seemed possible to choose permanent protecting groups such that their removal under basic conditions would keep the side reaction to acceptable levels (<30%).

Table 4.22.1 Recommended Permanent Protection Scheme[a,b]

	All nucleopeptides	For base-stable nucleopeptides only[c]
Nucleobases		
A	Bz	Bz
C	Ac	Bz
G	Dmf	*i*-Bu
T	—	—
Amino acids		
Asp, Glu	OFm	OFm
Asn, Gln	—	—[c]
Lys	Fmoc	Tfa
Arg	$(Fmoc)_2$	$(Fmoc)_2$
His[d]	H, Tos (Dnp)	Tos (Dnp)
Ser, Thr, Tyr[e]	Ac	Ac
Trp	For	For
Met	(O)[f]	(O)
Cys	S-*t*Bu[g,h]	—c
Other parameters		
Handle	HMFS	NPE
Cleavage conditions	NH_3/dioxane, room temperature[h]	NH_3/dioxane, 55°C *or* (i) TBAF, (ii) NH_3/dioxane, 55°C

[a]Refer to Figure 4.22.3 for abbreviations and structures of the amino acid protecting groups. Dash indicates no protecting group needed.

[b]Abbreviations for base protection: Ac, acetyl; Bz, benzoyl; Dmf, dimethylaminomethylene; *i*-Bu, isobutyryl.

[c]Homoserine or tyrosine nucleopeptides that do not contain Asn, Gln, or Cys.

[d]Histidine is introduced protected with the tosyl (Tos) group, which is removed prior to oligonucleotide elongation. Dnp-protected histidine must be used if His is placed at the C-terminal position. Dnp removal requires overnight ammonia/dioxane treatment at room temperature. For further comments on histidine protection, see Strategic Planning.

[e]These residues are incorporated unprotected when they are the linking amino acid and protected as indicated when they are not.

[f]The crude nucleopeptide must be treated with *N*-methylmercaptoacetamide to reduce the sulfoxide to a thioether.

[g]DTT must be added to the ammonia/dioxane solution to simultaneously remove the thiol protecting group.

[h]These very mild deprotection conditions must be used if serine or threonine are the linking residues or if the nucleopeptide contains asparagine and cysteine. In other circumstances, strongest deprotection treatments may be allowed (see Strategic Planning, discussion of final deprotection conditions).

All of the permanent protecting groups used in this procedure are shown in Table 4.22.1 and Fig. 4.22.3, and are discussed further below. They are chosen to be labile to the standard oligonucleotide-deprotecting reagent, aqueous ammonia. This is fully compatible with tyrosine nucleopeptides. Furthermore, if the nucleopeptide is not to reproduce the linking site of a naturally occurring nucleoprotein, the natural nonproteinogenic amino acid homoserine can be used as the linking residue (Fig. 4.22.2B), as first described in 1997 (Beltrán et al., 1997; Robles et al., 1997). No β-elimination can take place in this case, since the phosphate group is linked to the γ-carbon of the amino acid. Note, however, that the great tendency of homoserine to form γ-lactones precludes its use as the C-terminal amino acid. For the synthesis of serine and threonine nucleopeptides, the mildest final deprotection conditions must be used (see below).

Amino acid	Side chain	Protected side chain	Structure of the protecting groups
Asp, Glu	$-(CH_2)_{1\text{-}2}-CO-OH$	$-(CH_2)_{1\text{-}2}-CO-O-Fm$	Fm
Asn, Gln	$-(CH_2)_{1\text{-}2}-CO-NH_2$	—	
Lys	$-(CH_2)_4-NH_2$	$-(CH_2)_4-NH-Tfa$ $-(CH_2)_4-NH-Fmoc$	$-CO-CF_3$ Tfa
Arg	$-(CH_2)_3-NH-C(=NH)-NH_2$	$-(CH_2)_3-NH-C(=N-Fmoc)-NH-Fmoc$	$-CO-O-$ Fmoc
His	$-CH_2-$ (imidazole: N, NH)	$-CH_2-$ (imidazole: N, N)–Tos $-CH_2-$ (imidazole: N, N)–Dnp	$-SO_2-C_6H_4-CH_3$ Tos
Ser	$-CH_2-OH$	$-CH_2-O-Ac$	$-C_6H_3(NO_2)(O_2N)$ Dnp
Thr	$-CH(CH_3)-OH$	$-CH(CH_3)-O-Ac$	
Tyr	$-CH_2-C_6H_4-OH$	$-CH_2-C_6H_4-O-Ac$	$-CO-CH_3$ Ac
Trp	$-CH_2-$ (indole NH)	$-CH_2-$ (indole N–For)	$-C(=O)H$ For
Met	$-CH_2-CH_2-S-CH_3$	$-CH_2-CH_2-S(=O)-CH_3$	
Cys	$-CH_2-SH$	$-CH_2-S-S-t$Bu	

Figure 4.22.3 Structures of the unprotected and protected side chains of the trifunctional amino acids, and structures of the permanent protecting groups suitable for nucleopeptide synthesis. Abbreviations: Ac, acetyl; Dnp, 2,4-dinitrophenyl; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; For, formyl; Tfa, trifluoroacetyl; Tos, tosyl.

Solid Support

Copolymers of styrene and 1% divinylbenzene are classic supports for peptide synthesis, while controlled-pore glass beads are typically used for the preparation of oligonucleotides. Polyethyleneglycol-polystyrene copolymers have been used in the synthesis of both types of molecules. In the authors' hands, the best results have been achieved using the polystyrene-1%-divinylbenzene copolymer (Robles et al., 1999). The authors use the *p*-methylbenzhydrylamine resin (see Fig. 4.22.2A), but aminomethylpolystyrene should also work.

Internal Reference Amino Acid

Incorporation of an internal reference amino acid (IRAA; Fig. 4.22.2A) has two benefits. First, it allows one to adjust the substitution degree of the solid support to the desired level. Previous work (Montserrat et al., 1994) showed that substitution degrees of >0.20 mmol/g are not suitable for oligonucleotide synthesis, and that amine-functionalized polystyrene resins generally have higher loadings. The loading can be reduced by incompletely incorporating one amino acid and capping the unreacted amines. Use of N^{α}-Fmoc derivatives has the advantage that deprotection of an aliquot with piperidine and spectrophotometric quantitation of the resulting *N*-(9-fluorenylmethyl)piperidine easily allows the degree of substitution to be calculated.

The second benefit of incorporation of an IRAA is that it allows some reaction yields to be determined. On one hand, the yield of incorporation of the C-terminal amino acid can be assessed by the ratio of C-terminal amino acid to IRAA obtained after acid hydrolysis and amino acid analysis of an aliquot of resin. On the other hand, the comparison of the peptide/IRAA ratio before and after cleavage allows one to determine the cleavage yield. Obviously, the IRAA must be different from the nucleopeptide amino acids.

Handle

The handle permanently protects the carboxyl group of the C-terminal amino acid (Fig. 4.22.2A). It is a bifunctional molecule with one carboxyl and one hydroxyl group. The former forms a stable amide bond with the IRAA and the latter an ester with the C-terminal amino acid. Cleavage of the nucleopeptide-resin ester linkage requires basic conditions to which the nucleopeptide remains stable.

The authors have tested two different handles: 2-nitrophenyl ester (NPE; Eritja et al., 1991) and fluorenylmethyl ester (HMFS; Albericio et al., 2001). The fluorenylmethyl ester is more labile than the 2-nitrophenyl ester, and is therefore the most adequate to synthesize serine and threonine nucleopeptides. Since mild cleavage conditions are required to prevent peptide-related side reactions (as well as to prevent base-promoted peptide-oligonucleotide cleavage, see below), the HMFS handle is now routinely used in the authors' laboratory and is the recommended option.

Protecting Groups for the Peptide Chain

Peptide assembly is carried out using the *tert*-butoxycarbonyl (Boc) group to temporarily protect the α-amino function. Removal of this protecting group requires treatment with 30% to 40% trifluoroacetic acid (TFA) in dichloromethane, which is fully compatible with the base-labile ester that links the C-terminal amino acid to the resin. Protection of the trifunctional amino acid side chains is discussed for each type of residue (see Table 4.22.1 and Fig. 4.22.3).

Aspartic and glutamic acids

The β- and γ-carboxyl groups of these residues are protected as 9-fluorenylmethyl (Fm) esters, which have the same lability as the nucleopeptide-HMFS linkage. Boc-Asp(OFm)-OH and Boc-Glu(OFm)-OH are commercially available. Side reactions associated with aspartimide formation in some peptide sequences (most notably, Asp-Gly) may take place during the final deprotection treatment (Jeyaraj et al., 2002).

Asparagine and glutamine

The primary carboxamides of these residues' side chains can be left unprotected. Phosphitylation of these groups by tetrazole-activated phosphoramidites has been shown to be virtually nil (Garcia de la Torre et al., 1999; Debéthune et al., 2002a), so there is very little risk of obtaining branched nucleopeptides. Final deprotection must be carried out using the mildest conditions (see below) to prevent side reactions associated with the 55°C ammonia treatment of asparagine-containing nucleopeptides (Debéthune et al., 2002a).

Lysine

The ε-amino function can be protected with either the trifluoroacetyl (Tfa) or 9-fluorenyl-methoxycarbonyl (Fmoc) groups (Robles et al., 1999). Tfa can only be removed by ammonia treatment at 55°C, while milder basic treatments eliminate the Fmoc groups. Both derivatives are commercially available.

Arginine
The easiest way to prevent reactions at the guanidinium group is to keep it protonated. Washing with solutions of weak proton donors (e.g., 1-hydroxybenzotriazole, tetrazole) prior to amino acid or nucleoside coupling is required to ensure that the strongly basic guanidinium group is not free. Nevertheless, optimal results were achieved using the Boc-Arg(Fmoc)$_2$-OH derivative (Debéthune et al., 2002a). Boc-Arg-OH·HCl can be purchased from commercial suppliers and the synthesis of Boc-Arg(Fmoc)$_2$-OH is described in this unit (see Support Protocol 2).

Histidine
The imidazole ring must be protected during histidine incorporation to prevent racemization at the α-carbon. Two commercially available Boc-histidine derivatives can be used for nucleopeptide synthesis; they are imidazole-protected using either the tosyl (Tos) group or the 2,4-dinitrophenyl (Dnp) group (Beltrán et al., 1998). The tosyl group is labile to nucleopeptide deprotection conditions and can also be removed by treatment with 1-hydroxybenzotriazole (HOBt; Fujii and Sakakibara, 1974). Since the best-quality crude nucleopeptides were obtained when phosphoramidites were coupled onto histidine-unprotected peptide-resins, it is recommended that the experimenter (1) use Boc-His(Tos)-OH for peptide synthesis, (2) treat with HOBt to deprotect the imidazole ring (if no HOBt has been used at any amino acid coupling step), and (3) proceed with the oligonucleotide assembly.

In nucleopeptides with histidine at the C terminus, the imidazole ring should be kept protected throughout the entire synthesis process. This is best achieved using the Dnp group. Undesired detachment of the growing nucleopeptide chains from the resin, which can slowly take place if the imidazole ring is unprotected, can thus be avoided. The Dnp group is labile to fluoride treatment (see below) as well as ammonia treatment at either room temperature (overnight) or 55°C (6 hr).

Serine, threonine, and tyrosine
The side-chain hydroxyl of these amino acids (when they are not used as the linking residue) are protected with the acetyl (Ac) group (Fig. 4.22.3), which can be removed in the mildest final deprotection conditions. Boc-Ser(Ac)-OH and Boc-Tyr(Ac)-OH can be purchased from commercial suppliers. The preparation of Boc-Thr(Ac)-OH is described in this unit (see Support Protocol 5).

Tryptophan
Best results are achieved using the commercially available indole-protected derivative Boc-Trp(For)-OH, in which the indole ring is *N*-formylated (Robles et al., 1999). The formyl group can be removed by ammonia treatment at room temperature.

Methionine
The sulfide function of the methionine side chain can be alkylated during peptide elongation, and is oxidized at the nucleotide incorporation cycles. To prevent side reactions, the commercially available sulfoxide derivative Boc-Met(O)-OH is recommended (Marchán et al., 2000). After nucleopeptide synthesis and deprotection, the sulfoxide is reduced to a sulfide by reaction with *N*-methylmercaptoacetamide (see Support Protocol 6).

Cysteine

Boc-Cys(S-*t*Bu)-OH (where S-*t*Bu is *tert*-butylthio), in which the thiol group has been transformed into a sterically hindered disulfide, allows cysteine-containing nucleopeptides to be obtained (Debéthune et al., 2002a). Care must be taken during the final deprotection step to avoid loss of HS-S-*t*Bu by β-elimination. The target product is safely obtained if the final deprotection is carried out by treating the nucleopeptide-resin with ammonia at room temperature in the presence of 1,4-dithiothreitol (see Support Protocol 7). Boc-Cys(S-*t*Bu)-OH can be easily obtained from the commercially available H-Cys(S-*t*Bu)-OH derivative (see Support Protocol 3).

Linking Amino Acid

Serine, threonine, and tyrosine are introduced as N^{α}-Boc derivatives with the side-chain hydroxyl unprotected. No acylation of the free hydroxyl has been detected if the subsequent amino acids are incorporated using *N,N′*-dicyclohexylcarbodiimide (DCC) to activate the carboxyl group. N^{α}-Boc-protected homoserine derivatives are not available from commercial suppliers. The synthesis of the required compound is described here (see Support Protocol 4; Beltrán et al., 1997). This derivative, which is obtained as the triethylammonium salt Boc-Hse(DMTr)-O^- $HTEA^+$, will not couple onto the growing peptide chain unless a proton donor is added to the carbodiimide activator; therefore, this coupling is performed in the presence of HOBt.

Peptide Assembly

Peptide synthesis is carried out manually in polypropylene syringes fitted with polyethylene disks. An automatic synthesizer can also be used. As stated above, carbodiimides (*N,N′*-dicyclohexylcarbodiimide or *N,N′*-diisopropylcarbodiimide) are used to activate the carboxyl group of the incoming amino acid. 4-Dimethylaminopyridine must be added during the incorporation of the first (C-terminal) amino acid to achieve good esterification yields. Addition of HOBt is required to prevent side reactions during the activation and incorporation of asparagine, glutamine, and unprotected arginine, and to couple the homoserine derivative (HOBt can be replaced by tetrazole in this case).

Some peptide sequences are particularly prone to form diketopiperazines (Pro-aa or aa-Pro, where aa is any amino acid, are typical examples; Gisin and Merrifield, 1972). This means that the free amine group of a dipeptide reacts with the resin-linked terminal carboxyl, releasing a cyclic dipeptide (diketopiperazine) and liberating free hydroxyl groups on the solid support. This unwanted side reaction can be minimized (Gairí et al., 1990) by reacting the third amino acid with the trifluoroacetate salt of the dipeptide-resin in the presence of a strong carboxyl activator reagent (PyAOP, 7-aza-benzotriazol-1-yl-oxytripyrrolidinephosphonium hexafluorophosphate) and an excess of *N,N*-diisopropylethylamine.

Protecting Groups for the Oligonucleotide Moiety

Standard DMTr and 2-cyanoethyl groups are used for temporary protection of the 5′-hydroxyl (or 3′-hydroxyl, if 5′-phosphoramidites are used) and for permanent protection of the phosphates, respectively. For the preparation of tyrosine and homoserine nucleopeptides, and if no side reaction–originating amino acids (Asn, Asp, or Cys) are present, standard nucleobase-protecting groups can be used: benzoyl (Bz) for A and C, and isobutyryl (*i*-Bu) for G. If base-labile nucleopeptides are to be obtained and/or the hybrid contains “delicate” amino acids, more labile nucleobase-protecting groups must be used. C is then protected with the acetyl (Ac) group, and the exocyclic amine of G is protected as formamidine with the dimethylaminomethylene group (Dmf). The structures of all of these protecting groups appear in Figures 2.1.7 and 2.1.10.

Oligonucleotide Elongation

The oligonucleotide is assembled on an automatic DNA synthesizer (ABI 380B). Small modifications of the standard synthesis cycle are introduced to optimize performance with polystyrene resins (Bardella et al., 1990). The authors usually replace the standard iodine oxidation reagent with a 1 M solution of *t*-BuOOH, which facilitates washing of the resin after the oxidation step. Use of this reagent is absolutely mandatory when the nucleopeptide contains tyrosine or unprotected tryptophan, to prevent iodination of their aromatic side chains. Nucleopeptides with phosphorothioate internucleoside linkages have been obtained using the Beaucage reagent for the sulfurization step (Robles et al., 1999).

Final Deprotection Conditions

The polystyrene-divinylbenzene copolymer is hydrophobic and does not swell in aqueous medium. Consequently, treatments of nucleopeptide-resins with ammonia are not carried out using a 33% concentrated aqueous solution, but with a 1:1 (v/v) mixture of concentrated aqueous ammonia/dioxane.

To cleave [base-stable nucleopeptide]-resin bonds, a two-step procedure is recommended: (1) treatment with tetrabutylammonium fluoride in anhydrous tetrahydrofuran, which detaches the nucleopeptide from the resin and removes most amino acid–protecting groups, and (2) treatment of the resulting filtrate with concentrated ammonia (or 1:1 ammonia/dioxane) to eliminate other remaining protecting groups. The authors have obtained better cleavage yields with this procedure than with an overnight treatment of the nucleopeptide-resin with ammonia at 55°C. The reaction with fluorides is a β-elimination process that yields the nucleopeptide with a C-terminal carboxylic acid function (Fig. 4.22.4A). The tyrosine-nucleoside phosphodiester bond remains unaffected by fluoride treatment.

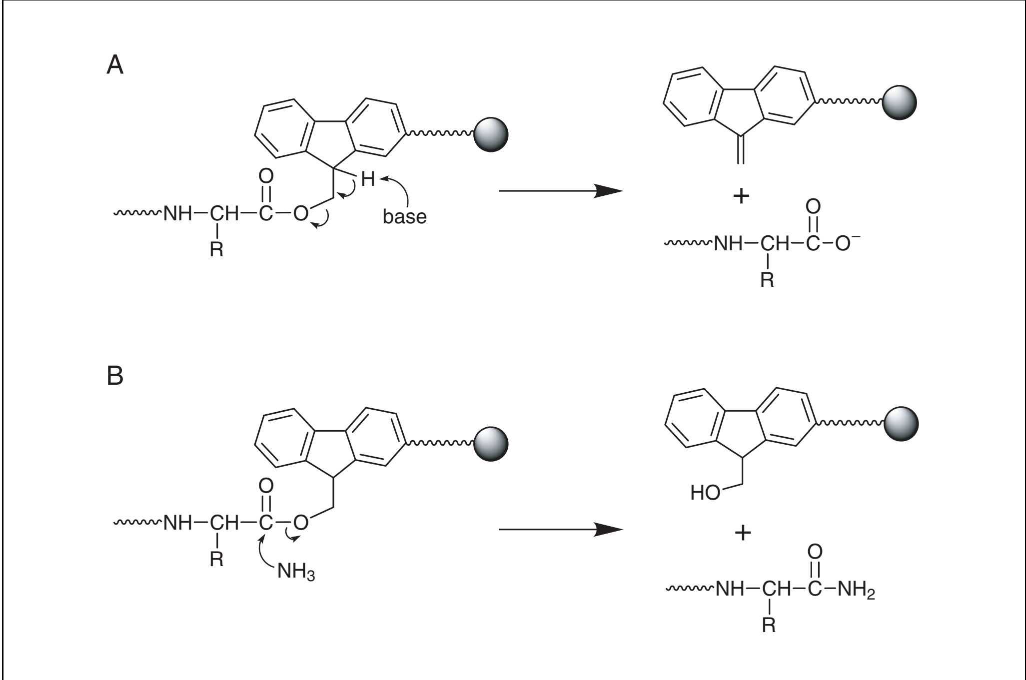

Figure 4.22.4 Mechanisms of cleavage of the [C-terminal amino acid]-resin ester linkage. (**A**) Base-promoted β-elimination yields a C-terminal carboxylic acid function; (**B**) nucleophile-promoted cleavage yields a C-terminal carboxamide.

The nucleopeptide-HMFS linkage can also be cleaved under milder conditions by treatment with 1:1 ammonia/dioxane at room temperature. Although cleavage yields are lower than if the reaction is carried out at 55°C, this is the only alternative if the target nucleopeptide has serine or threonine as the linking residue, or if it contains amino acids (or peptide sequences) which are prone to give side reactions under these conditions (see above).

An additional point of concern when the nucleopeptide-resin is treated directly with ammonia is that cleavage can take place either by β-elimination (Fig. 4.22.4A), which affords the nucleopeptide acid, or by nucleophilic attack of ammonia at the ester bond between the C-terminal amino acid and the HMFS handle (Fig. 4.22.4B). Nucleopeptides are then obtained as C-terminal carboxamides. Acid/carboxamide mixtures (in which the acid is the main component) are always obtained when glycine is the C-terminal amino acid (Debéthune et al., 2002a). The amount of amide is virtually nil when other residues occupy the C-terminal position.

Summary

As a general rule, the authors recommend assembling the nucleopeptide using a set of permanent protecting groups that can be removed using the mildest deprotection conditions, but little or no care has to be taken if tyrosine or homoserine is the linking amino acid and the nucleopeptide does not include "delicate" amino acids.

However, even under the mildest possible final deprotection conditions, degradation of serine nucleopeptides by base-mediated cleavage of the serine-nucleoside phosphodiester bond can take place in some cases (Debéthune et al., 2002a). The data available indicate that this is a sequence-dependent reaction whose extent cannot as yet be predicted.

From an operational point of view, the preparation of a nucleopeptide requires an adequately functionalized solid support and suitably protected amino acid and nucleoside derivatives. Access to an HPLC, a UV spectrophotometer, an electrospray or MALDI-TOF mass spectrometer, and an amino acid analyzer is required. Denaturing polyacrylamide gel electrophoresis (PAGE) facilities may also be necessary.

STEPWISE MANUAL SOLID-PHASE SYNTHESIS OF THE PEPTIDE

BASIC PROTOCOL 1

In this protocol, the C-terminal amino acid is first coupled to a solid support that has already been coupled with the internal reference amino acid (IRAA) and the HMFS linker (see Support Protocol 1 and Fig. 4.22.2A). Incorporation of the terminal amino acid is compared to the IRAA by amino acid analysis. After acylation of unreacted HMFS hydroxyl groups, the subsequent amino acids are added until the final support-bound peptide has been synthesized (Fig. 4.22.2B).

During synthesis, each coupling is monitored by a ninhydrin or chloranil test. The ninhydrin test (Kaiser et al., 1970) is only reliable for primary amines. For a qualitative coupling assay in the case of proline, the chloranil test (Christensen, 1979) should be used to assay for secondary amines.

In general, the support-bound peptide (step 28) can be used directly for elongation of the oligonucleotide chain (see Basic Protocol 2). In some cases, especially with long peptides, analysis of the deprotected crude peptide by HPLC and mass spectrometry techniques (steps 29 to 41) is recommended to ensure peptide homogeneity before oligonucleotide elongation.

Materials

Derivatized *p*-methylbenzhydrylamine solid support (H-HMFS-IRAA-MBHA-PS; see Support Protocol 1)
Dichloromethane (DCM; peptide synthesis grade)
N,N-Dimethylformamide (DMF; peptide synthesis grade)
t-Butoxycarbonyl-protected amino acids (Boc-aa-OH):
Ala, Arg·HCl, Asn, Asp(OFm), Gln, Glu(OFm), Gly, His(Dnp), His(Tos), Ile, Leu, Lys(Fmoc), Lys(Tfa), Met(O), Phe, Pro, Ser, Ser(Ac), Thr, Trp(For), Tyr, Tyr(Ac), and Val (Novabiochem, Bachem, Neosystem)
Boc-Arg$(Fmoc)_2$-OH (see Support Protocol 2)
Boc-Cys(S-*t*Bu)-OH (see Support Protocol 3)
Boc-Hse(DMTr)-$O^-HNEt_3^+$ (see Support Protocol 4)
Boc-Thr(Ac)-OH (see Support Protocol 5)
N,N′-Dicyclohexylcarbodiimide (DCC) or *N,N′*-diisopropylcarbodiimide (DiPC)
4-Dimethylaminopyridine (DMAP)
Methanol (MeOH; HPLC grade)
Concentrated HCl, analytical grade
Propionic acid
Phenol, crystalline
3 M aqueous *p*-toluenesulfonic acid containing 0.2% tryptamine
0.06 M citrate buffer, pH 2
Acetic anhydride
Benzoyl chloride (optional)
Pyridine, synthesis grade
30% (v/v) trifluoroacetic acid (TFA; peptide synthesis grade) in DCM
5% (v/v) *N,N*-diisopropylethylamine (DIPEA) in DCM
1-Hydroxybentotriazole (HOBt; for coupling unprotected Arg, Asn, Gln, Hse)
0.5 M HOBt in DCM (for coupling unprotected Arg)
(Azabenzotriazole-1-yl-oxy-tris-pyrrolidino)phosphonium hexafluorophosphate (PyAOP; for coupling any aa onto Pro)
N,N-Diisopropylethylamine (DIPEA)
Ethyl acetate (EtOAc)
1 M sulfuric acid
Sodium sulfate (Na_2SO_4), anhydrous
Ninhydrin reagents A and B (see recipes)
Saturated chloranil solution: ~0.75 g of 2,3,5,6-tetrachloro-1,4-benzoquinone in 25 mL toluene
Acetone
Peroxide-free dioxane (see recipe)
1 M 1,4-dithiothreitol (DTT) in peroxide-free dioxane (for S-*t*Bu-protected Cys)
32% (v/v) ammonium hydroxide (store at 4°C)
HPLC mobile phase A: 0.045% TFA in water (HPLC grade)
HPLC mobile phase B: 0.036% TFA in acetonitrile (HPLC grade)
α-Cyano-4-hydroxycinnamic acid (CHA) or dihydroxybenzoic acid (DHB) matrix
0.1% TFA in 1:1 (v/v) acetonitrile/water (HPLC grade)

2- to 10-mL disposable polypropylene syringes with porous polyethylene discs and Teflon two-way stop cocks (RTV SF2, Shimadzu Scientific Research)
Vacuum filtration system
Teflon stir rod
Desiccator
Pyrex tubes for amino acid analyses
Methane/oxygen flame

110° and 155°C heating block
Rotary evaporator equipped with a water aspirator or vacuum pump (use aspirator unless pump is indicated)
0.45-μm nylon filters
Automatic amino acid analyzer (Beckman System 6300)
25-mL separatory funnel
Filter paper
25- and 50-mL round-bottom flasks
Small glass tubes for ninhydrin or chloranil tests
4-mL screw-cap pressure tubes, O-ring seal preferred

Additional reagents for HPLC and MALDI-TOF-MS (see Basic Protocol 3 and Chapter 10)

CAUTION: Trifluoroacetic acid is a very corrosive acid. Breathing the vapors is very harmful and it is possible to be quickly overcome by them. Always manipulate with extreme caution in a well-ventilated fume hood and wear appropriate protective clothing.

Prepare syringe with solid support for peptide synthesis

1. Transfer an accurately weighed amount of H-HMFS-IRAA-MBHA-PS solid support for a 50-μmol synthesis into a 5- to 10-mL polypropylene syringe fitted with a porous polyethylene disc and equipped with a Teflon two-way stop cock.
2. Place the syringe on a vacuum filtration system.

Incorporate C-terminal amino acid onto support

3. Wash the solid support using three 30-sec washes each of DCM, DMF, and then DCM again.

   For washes and for deprotection and neutralization treatments, a solvent volume of one or two times the volume of resin is recommended. For coupling, it is important to keep the reaction mixture as concentrated as possible. Coupling reactions must therefore be carried out in the minimum amount of solvent required to swell the resin and allow stirring.

   It is important to handle the polystyrene resin carefully to avoid mechanical degradation of the solid matrix. In all steps that require mixing, the resin should be stirred very carefully with a Teflon stir rod.

4. Weigh out the required amount of Boc-aa-OH (10 eq), DCC (10 eq), and DMAP (0.5 eq), and dissolve the mixture in a minimal volume of 1:1 (v/v) DCM/DMF.

   DiPC can replace DCC in all reactions described in this protocol and in the Support Protocols. The urea derivative formed after activation of the carboxyl group is more soluble in organic solvents.

   The esterification conditions employed for the incorporation of the C-terminal amino acid may cause some racemization, especially with amino acids such as unprotected histidine. In order to avoid this side reaction, glycine has been selected in most cases as the C-terminal amino acid. However, some problems may arise during the ammonium hydroxide deprotection (see Strategic Planning for more details).

5. Add the reaction mixture to the solid support and let stand 1.5 hr, occasionally stirring with a Teflon stir rod.
6. Wash the solid support successively with three 30-sec washes of DMF and three 30-sec washes of DCM.
7. Repeat steps 4 to 6 using 5 eq Boc-aa-OH, 5 eq DCC, and 0.25 eq DMAP and a reaction time of 1 hr.
8. Wash the solid support three times for 30 sec with MeOH and dry in a desiccator.

Determine C-terminal amino acid incorporation: General procedure for amino acid analysis

9. Withdraw a 5- to 10-mg aliquot of dry resin and place it in a Pyrex tube.

10. Add 300 µl of 1:1 (v/v) concentrated HCl/propionic acid to the resin.

    When tyrosine is present, add a phenol crystal to the mixture to prevent decomposition of this amino acid during acid hydrolysis.

    Some amino acids, such as cysteine, homoserine, serine, threonine, and tryptophan, are partially or completely destroyed during acid hydrolysis. Arginine is partially converted to ornithine, and asparagine and glutamine to aspartic and glutamic acids, respectively. When the determination of some of these particular residues is necessary, enzymatic digestion must be carried out (see Basic Protocol 3, step 51). When determining tryptophan incorporation, hydrolysis must be carried out with a 3 M p-toluenesulfonic acid solution containing 0.2% of 3-(2-aminoethyl)indole (tryptamine), for 12 hr at 110°C in a vacuum-sealed Pyrex tube.

11. Close the Pyrex tube hermetically using a methane/oxygen flame and heat 1.5 hr in a 155°C heating block.

12. Cool to room temperature, open the Pyrex tube, and evaporate the acidic mixture to dryness in a rotary evaporator equipped with a vacuum pump.

    CAUTION: *Always wear gloves and glasses and work in a well-ventilated fume hood when carrying out these operations. Be careful when closing and opening the tube.*

13. Redissolve the dry residue in 500 µL of 0.06 M citrate buffer, pH 2, and filter the resulting solution through a 0.45-µm nylon filter. Inject in an automatic amino acid analyzer.

    The amount of sample necessary for carrying out the amino acid analysis depends on the analyzer. For some analyzers, 5 nmol amino acid is optimal. In that case, injection of 50 µL of the resulting solution will give 2 to 10 nmol of each residue.

    Prior to analyzing the sample, a standard that contains all 20 natural amino acids is analyzed to calculate a response factor for each residue under the analysis conditions employed. The response factors are used to calculate the amount of each residue in the sample.

14. Compare incorporation of the C-terminal amino acid to incorporation of the IRAA. If (C-terminal amino acid/IRAA) < 0.9, repeat steps 7 to 13. If $0.9 \leq$ (C-terminal amino acid/IRAA) ≤ 1, proceed with step 15.

    In general, a nearly quantitative incorporation is achieved.

Acylate unreacted hydroxyl groups of HMFS linker

15. Add the required amount of acetic anhydride or benzoyl chloride (10 eq), pyridine (10 eq), and DCM to the solid support and let stand 10 min with occasional stirring using a Teflon rod.

    As for the coupling steps, a minimum volume of DCM is used for this step. The volume should be sufficient to swell the resin and allow stirring with the Teflon rod.

16. Wash the resin three times for 30 sec with DCM and repeat steps 15 and 16 to ensure that all unreacted hydroxyl groups are capped.

17. Wash the resin successively with three 30-sec washes each of DMF, DCM, and MeOH. Dry directly on a vacuum filtration system.

Incorporate subsequent amino acids

18. Add the required volume of 30% (v/v) TFA in DCM (one or two times the resin volume) and let stand 5 min with occasional stirring using a Teflon rod.

19. Wash with DCM, add an equal volume of 30% TFA in DCM, and let stand 25 min with occasional stirring.

20. Wash thoroughly with DCM, then wash four times for 60 sec with 5% (v/v) DIPEA in DCM, and five times for 30 sec with DCM.

21. Weigh out the required amount of Boc-aa-OH (3 eq) and DCC (3 eq), and dissolve the mixture in the minimum quantity of 1:1 (v/v) DCM/DMF. Add to the solid support and let stand 1.5 hr with occasional stirring using a Teflon rod. Modify as necessary for the following amino acid–specific conditions.

    When coupling arginine with the side chain protonated, weigh out the required amount of Boc-Arg-OH·HCl·2H_2O (3 eq), DCC (3 eq), and HOBt (6 eq), and dissolve the mixture in the minimum amount of 1:1 (v/v) DCM/DMF. To avoid undesired side reactions, wash the resin four times for 60 sec with 0.5 M HOBt in DCM between steps 20 and 21.

    For asparagine or glutamine, weigh out the required amount of Boc-Asn-OH or Boc-Gln-OH (3 eq), DCC (3 eq), and HOBt (3 eq), dissolve in the minimum quantity of 1:1 (v/v) DCM/DMF, and let stand 10 min at 0°C before adding it to the resin.

    When coupling the second amino acid onto Pro-resin, skip the neutralization step with 5% (v/v) DIPEA in DCM (step 20), weigh out the required amount of Boc-aa-OH (5 eq) and PyAOP (5 eq), dissolve the mixture in the minimum quantity of 1:1 (v/v) DCM/DMF, and add it to the resin. Then add 10 eq DIPEA to the resin and let stand for 1.5 hr, stirring occasionally with a Teflon rod.

    An acidic additive is necessary for the incorporation of the triethylammonium salt of the homoserine derivative. Weigh out the required amount of Boc-Hse(DMTr)-$O^-HNEt_3^+$ (3 eq), DCC (3 eq), and HOBt (3 eq), and dissolve the mixture in the minimum quantity of 1:1 (v/v) DCM/DMF. After incorporation onto the support, elimination of the tert-butoxycarbonyl group with trifluoroacetic acid removes the DMTr group, so it is possible to quantify the coupling yield by quantitating the released DMTr cation (see Basic Protocol 2, step 11).

    When using Boc-Thr(Ac)-O^-DCHA^+, a workup is necessary to isolate the protected amino acid in the neutral species. Suspend Boc-Thr(Ac)-O^-DCHA^+ in EtOAc (~5 mL/mmol amino acid) and transfer to a 25-mL separatory funnel. Add the same volume of 1 M sulfuric acid. Shake to achieve complete dissolution of the amino acid derivative. Separate the organic layer, and extract the aqueous phase with more EtOAc (twice with 5 mL). Dry the organic layer over anhydrous Na_2SO_4. Add more Na_2SO_4 if the salt crystals clump together upon swirling. Gravity filter the resulting solution through filter paper into a 50-mL round-bottom flask. Rinse the Na_2SO_4 crystals with 5 to 10 mL EtOAc. Remove the solvent under reduced pressure (i.e., in a rotary evaporator) to yield a white solid. Perform several co-evaporations with DCM to eliminate traces of EtOAc.

22. Wash the resin successively with three 30-sec washes of DMF, DCM, and MeOH. Dry directly on a vacuum filtration system.

Assess amino acid coupling

23. Withdraw a 0.5- to 1-mg aliquot of dry resin and place it in a small glass tube.

24a. *For primary amines:* Add 3 drops of ninhydrin reagent A and 1 drop of ninhydrin reagent B. Heat the tube 3 min in a 110°C heating block. Allow to cool to room temperature. Assess color and proceed to step 25.

    It is important to have good quality ninhydrin reagents in order to obtain a reliable result. See Reagents and Solutions for more specific details. This test was developed for the primary amines of amino acids and may not be reliable for other primary amines.

24b. *For proline:* Add 5 drops saturated chloranil solution and 20 drops acetone. Stir 5 min at room temperature. Assess color and proceed to step 25.

25a. *For negative tests:* If the reaction mixture is yellow (indicative of a successful amino acid coupling), proceed with the incorporation of the next amino acid (step 26).

25b. *For positive tests:* If the reaction mixture is blue for ninhydrin or green-blue for chloranil (indicative of the presence of free primary or secondary amines, respectively), repeat the coupling of the amino acid using the same amounts and conditions (steps 18 to 22) and then repeat the ninhydrin or chloranil test.

25c. *For repeated positive tests:* If a blue or green-blue color persists after a second coupling of the amino acid, cap the unreacted amines by adding acetic anhydride (10 eq), DIPEA (10 eq), and DMF to the solid support. Let stand 10 min, stirring occasionally with a Teflon rod. Wash the resin sequentially using three 30-sec washes each of DMF followed by DCM. Repeat the ninhydrin or chloranil test to confirm that all unreacted amines are capped before proceeding with the incorporation of the next amino acid (step 26).

26. Perform incorporation of the second and subsequent amino acids (steps 18 to 22), confirming the success of each coupling (steps 23 to 25) before proceeding to the next.

    Some minor modifications have to be introduced depending on the desired functional group at the N-terminal position of the peptide. If the amino group has to be acylated, remove the tert-butoxycarbonyl group and acylate by treating with acetic anhydride (3 eq) in DMF for 15 to 25 min. A longer treatment could cause undesired acetylation of the free side chain of the hydroxylated amino acid. When a free N-terminal position is desired, incorporate the last residue as an Fmoc-protected amino acid instead of a Boc derivative. The fluorenylmethoxycarbonyl group will be removed during the final ammonium hydroxide treatment. In some cases the tert-butoxycarbonyl group can be used to block the N-terminal position of the peptide. If that is the case, no further modification is necessary to the standard procedure because the Boc group is stable to the final ammonium hydroxide treatment.

27. Once the final (N-terminal) amino acid has been successfully coupled, proceed with the amino acid analysis as described in steps 9 to 13.

    When an Fmoc-amino acid derivative is introduced at the N-terminal position of the peptide, it is recommended to eliminate the Fmoc group before carrying out acid hydrolysis. This is because the Fmoc group may not be completely removed during the acid hydrolysis treatment, especially when it protects some sterically hindered residues such as isoleucine. For removal of the Fmoc group, treat the resin aliquot with 20% (v/v) piperidine in DMF as described below (see Support Protocol 1).

28. Calculate the relative ratio of the amino acids according to their peak areas and response factors (see step 13).

    If the peptide was correctly elongated, the ratio of residues will be close to the theoretical value.

Perform cleavage and deprotection (optional)

29. Take a 5- to 10-mg aliquot of dry peptide-resin and place it in a 4-mL screw-cap pressure tube, preferably with an O-ring seal.

30. Add 1 mL peroxide-free dioxane and 1 mL of 32% ammonium hydroxide solution, screw the cap on tightly, and leave 6 hr at room temperature.

    If the peptide sequence contains trifluoroacetyl-protected lysine, perform the deprotection at 55°C for 12 to 15 hr. If it contains tert-butylthio-protected cysteine, use 1 mL of 1 M 1,4-dithiothreitol in dioxane and 1 mL of 32% ammonium hydroxide.

Table 4.22.2 Typical Gradient and Mobile Phase for RP-HPLC of Deprotected Crude Peptide[a]

Elapsed time (min)	Percent mobile phase B[b]
0	10
30	50
31	100
35	100
36	10
45	10

[a]Gradient conditions are based on a flow of 1 mL/min using a Kromasil reversed-phase C18 column (5-µm-diameter spherical silica, 4.0 × 250 mm) at room temperature with a 45-min overall cycle. Mobile phase A: 0.045% trifluoroacetic acid (TFA) in water; mobile phase B: 0.036% TFA in acetonitrile.
[b]Percentage is at elapsed time.

31. Filter through a 2-mL polypropylene disposable syringe with a polyethylene or cotton filter and collect the solution in a 25-mL round-bottom flask.

32. Wash the pressure tube several times with MeOH and pass through the syringe.

    With tert-butylthio-protected cysteine-containing peptides, removal of 1,4-dithiothreitol is required prior to HPLC analysis. Dilute the crude sample to 2 mL by adding water and wash three times with 2 mL EtOAc. Proceed with the aqueous phase.

33. Concentrate the combined solution under reduced pressure to a volume of ~0.5 mL. Dilute the deprotected crude peptide with 0.5 mL HPLC mobile phase B and take an aliquot (10 to 50 µL) for HPLC analysis.

Analyze crude peptide by HPLC (optional)

34. Program the gradient system to start with 10% mobile phase B, increasing the percentage of mobile phase B with time (Table 4.22.2).

    Ensure that there is sufficient mobile phase to keep intakes covered throughout the run. For more specific details about HPLC analysis, see Basic Protocol 3 and UNIT 10.5.

35. Set the UV detector wavelength at 220 nm. Equilibrate the HPLC system with the starting mobile phase composition (10% mobile phase B) until a flat baseline is achieved at this detection wavelength.

36. Inject the sample by programming the autosampler or by loading it into the sample loop using the appropriate syringe. Elute the sample by starting the gradient. Record the chromatogram.

    Typically, small peptides will elute at ~10 to 15 min under the recommended conditions (Table 4.22.2). When long peptides are prepared, it is recommended to use a gradient from 10% to 70% or 80% mobile phase B over 30 min and then wash as usual.

    Ammonium hydroxide treatment gives a mixture of the C-terminal carboxylic acid and carboxamide peptides, which usually elute in this order. Some UV-absorbing compounds are eluted at high acetonitrile percentages. These peaks are associated with handle-derived products formed during ammonium hydroxide treatment.

Analyze crude peptide by MALDI-TOF-MS (optional)

37. Weigh 10 mg matrix (CHA or DHB) and place in a 1.5-mL microcentrifuge tube.

    A CHA matrix is recommended for small peptides (mol. wt. <500) and DHB for a wide range of masses.

38. Add 1 mL of 0.1% TFA in 1:1 (v/v) acetonitrile/water to the matrix. Vortex 30 sec to allow complete dissolution.

    Optimal results are obtained when the highest-purity matrices are used. Matrix solutions should be made fresh prior to use. Storage for more than 3 days is not recommended.

39. To a 0.5-mL microcentrifuge tube, add 1 µL peptide sample solution and 1 µL matrix solution. Mix by withdrawing and expelling the solution ten times with the pipet.

40. Spot 1 µL solution on a MALDI-TOF sample plate and dry. To allow homogeneous crystallization, do not disturb the spotted samples after they start to crystallize.

41. Perform peptide MALDI-TOF-MS analysis in positive mode.

SUPPORT PROTOCOL 1

PREPARATION OF THE SOLID SUPPORT (H-HMFS-IRAA-MBHA-PS)

This protocol describes the coupling of the internal reference amino acid and handle to the solid support used for nucleopeptide synthesis (see Fig. 4.22.2A).

Additional Materials *(also see Basic Protocol 1)*

p-Methylbenzhydrylamine resin: (α-amino-α-xylyl)-polystyrene reticulated with 1% divinylbenzene (*f* = 0.5 to 0.6 mmol/g; Novabiochem)
9-Fluorenylmethoxycarbonyl-protected internal reference amino acid (Fmoc-aa; Novabiochem, Bachem, Neosystem)
20% (v/v) piperidine in DMF
N-[9-(Hydroxymethyl)-2-fluorenyl]succinamic acid (H-HMFS-OH; Alberico et al., 2001)

2- and 10-mL polypropylene syringes fitted with polyethylene discs
Teflon two-way stopcocks
50-mL volumetric flasks
Double-beam UV spectrophotometer, calibrated, and quartz cuvettes

Wash solid support

1. Transfer an accurately weighed amount of *p*-methylbenzhydrylamine resin into a polypropylene syringe fitted with a polyethylene disc (i.e., 250 mg of resin into a 10-mL syringe).

2. Place the syringe on a vacuum filtration system.

3. Wash the solid support with three 30-sec washes of DCM.

    For general guidelines on volumes and handling resin, see Basic Protocol 1, step 3.

4. Add the required volume of 30% (v/v) trifluoroacetic acid in DCM and let stand 5 min, occasionally stirring with a Teflon rod.

5. Wash with DCM, add an equal volume of 30% (v/v) trifluoroacetic acid in DCM, and let stand 25 min.

6. Wash sequentially as follows:

    Three times for 30 sec with DCM
    Four times for 60 sec with 5% (v/v) DIPEA in DCM
    Five times for 30 sec with DCM.

Incorporate internal reference amino acid

7. Weigh out the required amount of Fmoc-aa (0.5 eq) and DCC (0.5 eq), and dissolve the mixture in the minimum quantity of 1:1 (v/v) DCM/DMF.

   The internal reference amino acid must not be present in the target nucleopeptide sequence.

8. Add the reaction mixture to the solid support and let stand 25 min, stirring occasionally with a Teflon rod.

   Coupling time and amount of reactants are optimized to obtain a desired loading of ≤0.25 mmol/g. An increased coupling time will yield higher substitution degrees that are not adequate for oligonucleotide elongation.

9. Wash the solid support sequentially using three 30-sec washes each of DMF followed by DCM.

Determine loading

10. Withdraw a 10-mg aliquot of dry resin and place it in a 2-mL polypropylene syringe fitted with a polyethylene disc and stopcock.

11. Add 1 mL of 20% (v/v) piperidine in DMF and let stand 3 min, occasionally stirring with a Teflon rod.

12. Open the stopcock and collect the filtrate in a 50-mL volumetric flask. Wash three times with 2 mL DCM and collect in the same flask.

13. Repeat the piperidine treatment for 7 min and wash again three times with DCM. Collect these filtrates together with the previous ones, and dilute with DCM to the 50-mL mark.

    Piperidine treatments quantitatively remove the fluorenylmethoxycarbonyl protecting group. An N-(9-fluorenylmethyl)piperidine (Fmp) adduct is formed during this treatment. Its molar extinction coefficient at 300 nm (ε_{300}) is 7800. Its quantitation allows the loading of the solid support to be determined.

14. Prepare a reference solution by adding 2 mL of 20% (v/v) piperidine in DMF to a 50-mL volumetric flask and diluting to the mark with DCM.

15. Measure the sample absorbance at 300 nm (A_{300}) on a calibrated, double-beam UV spectrophotometer using the above solution as a reference. If A_{300} >1, dilute sample as necessary to achieve a value <1.

    If using a single-beam spectrophotometer, a baseline should be run with a sample containing the reference solution (blank).

16. Calculate the solid support loading (f) as:

$$f\ (\text{mmol/g}) = \frac{A \times V \times 1000}{\varepsilon \times l \times m}$$

Equation 4.22.1

where V is the total volume of the solution (in mL), ε is the molar extinction coefficient of the Fmp adduct at 300 nm (7800 L/mol/cm), l the absorbance path of the cuvette (in cm), and m is the amount of solid support (in mg).

17. If $f \approx 0.15$ to 0.25 mmol/g, proceed according to step 18 with the entire support batch. If it is lower, repeat steps 7 to 9 with shorter coupling times.

Acylate unreacted amine groups and remove Fmoc

18. Add the required amount of acetic anhydride (10 eq), DIPEA (10 eq), and DMF to the solid support and let stand 10 min, stirring occasionally with a Teflon rod.

19. Wash the resin three times for 30 sec each with DCM and then repeat step 18. Perform the ninhydrin test with 0.5 to 1 mg of resin (see Basic Protocol 1, step 24a) to be sure that all unreacted amino groups are capped (solution should be yellow).

20. Wash the resin three times for 30 sec each with DMF, then DCM, and then MeOH. Dry directly on the vacuum filtration system.

21. Add the required volume of 20% (v/v) piperidine in DMF and leave 3 min, stirring occasionally with a Teflon rod.

22. Wash five times for 30 sec each with DCM and once again add the required volume of 20% (v/v) piperidine in DMF. Let stand 10 min, stirring occasionally with a Teflon rod.

23. Wash thoroughly three times for 30 sec each with DMF and then DCM. Dry directly on the vacuum filtration system.

Incorporate handle

24. Weigh out the required amount of H-HMFS-OH (3 eq) and DCC (3 eq) and dissolve the mixture in the minimum quantity of 1:1 (v/v) DCM/DMF.

    The synthesis of N-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid is carefully described elsewhere (see Albericio et al., 2001).

25. Add the reaction mixture to the solid support and let stand for 4 to 5 hr, stirring occasionally with a Teflon rod.

26. Wash the solid support successively (three times for 30 sec each) with DMF, then DCM, and finally MeOH. Dry directly on the vacuum filtration system.

27. Withdraw a 0.5- to 1-mg aliquot of dry resin and carry out the ninhydrin test (see Basic Protocol, step 24a). If the coupling is not complete (i.e., solution is blue, not yellow), repeat the handle incorporation using 1.5 eq for 1 to 2 hr.

SUPPORT PROTOCOL 2

PREPARATION OF Boc-Arg(Fmoc)$_2$-OH

Fmoc protection of Boc-Arg-OH is shown in Figure 4.22.5.

Materials

N-(*tert*-Butoxycarbonyl)-L-arginine hydrochloride (Novabiochem)
Acetonitrile, anhydrous (see recipe)
9-Fluorenylmethyl chloroformate (Fmoc·Cl)
Argon (Ar), dry
Dichloromethane (DCM, anhydrous; see recipe) in a septum-sealed distillation collection bulb
N,*N*-Diisopropylethylamine (DIPEA), anhydrous (see recipe)
Chlorotrimethylsilane
Dichloromethane (DCM; synthesis grade)
Sodium sulfate (Na_2SO_4), anhydrous
Methanol (MeOH)
Acetic acid (AcOH)
Hexanes
230- to 400-mesh silica gel

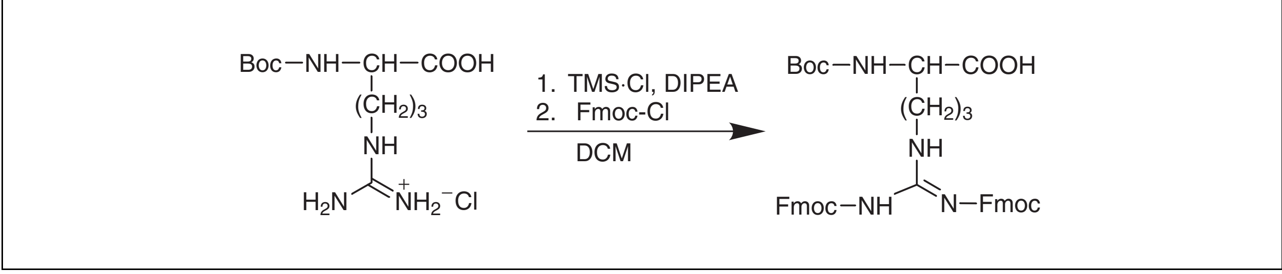

Figure 4.22.5 Protection of the guanidinium group of Boc-arginine. Abbreviations: Boc, *tert*-butoxycarbonyl; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; TMS, trimethylsilyl.

250-mL oven-dried round-bottom flask and rubber septum
Desiccator containing P_2O_5
Glass syringe and needle, oven dried
40°C heat block
250-mL separatory funnel
Gravity and vacuum filtration devices and filter paper
500-mL round-bottom flasks
2 × 5–cm silica-coated thin-layer chromatography (TLC) plates
254-nm UV light source
Rotary evaporator with a water aspirator
5 × 25–cm glass chromatography column with solvent reservoir bulb

Additional reagents and equipment for TLC (*APPENDIX 3D*), column chromatography (*APPENDIX 3E*), NMR, IR, and mass spectrometry

Fmoc-protect Boc-Arg-OH

1. Place 1.0 g (3.04 mmol) of *N*-(*tert*-butoxycarbonyl)-L-arginine hydrochloride in a 250-mL oven-dried round-bottom flask.

2. Co-evaporate three times with anhydrous acetonitrile.

3. Weigh 2.4 g (9.28 mmol) of Fmoc·Cl and dry overnight under vacuum in a desiccator containing P_2O_5.

4. Cap the flask with a rubber septum and purge the flask with dry Ar.

5. Withdraw anhydrous DCM from a septum-sealed distillation collection bulb using an oven-dried glass syringe and needle. Add 25 mL DCM to the purged flask with magnetic stirring.

6. Add 1.6 mL (9.4 mmol) anhydrous DIPEA and slowly add 1.5 mL (11.9 mmol) chlorotrimethylsilane.

7. Stir the reaction mixture 90 min at 40°C.

   The original suspension slowly becomes a solution.

8. Cool in an ice bath. Dissolve 2.4 g (9.28 mmol) Fmoc·Cl in a minimal amount of dry DCM. Add 1.6 mL DIPEA and the Fmoc·Cl to the cooled reaction.

9. Stir the mixture 20 min in the ice bath and then 6.5 hr at room temperature.

Work up reaction

10. Transfer the reaction mixture into a 250-mL separatory funnel and wash with 50 mL water. Wash the aqueous layer three times each with 50 mL DCM.

11. Dry the combined organic layer over anhydrous Na_2SO_4. Add more Na_2SO_4 if the salt crystals clump together upon swirling.

    When the solution is dry, the nonhydrated crystals will float in solution upon swirling.

12. Gravity filter the resulting solution through filter paper into a 500-mL round-bottom flask. Rinse the Na_2SO_4 crystals with 10 to 20 mL DCM.

13. Remove the solvent under reduced pressure (i.e., in a rotary evaporator with water aspirator) to yield an orange oil.

Monitor reaction by TLC

14. Dissolve an aliquot of oil in a minimal amount of DCM, spot the solution onto a precut 2 × 5–cm TLC plate, and develop using 93:5:2 (v/v/v) DCM/MeOH/acetic acid (APPENDIX 3D).

    CAUTION: *Wear protective eyewear.*

15. Visualize the spots under a 254-nm UV light source.

    TLC analysis should indicate a main product with R_f = 0.54 that corresponds to the desired amino acid derivative. Minor amounts of fast-moving products are usually present in the mixture.

Isolate and characterize product

16. Dissolve the orange oil in 1.5 to 2 mL DCM and pour the solution onto 30 mL hexanes.

    A yellowish precipitate is obtained. This procedure partially eliminates the less polar contaminants.

17. Filter the precipitate under vacuum.

18. Prepare a 5 × 25–cm glass chromatography column with solvent reservoir bulb by adding a slurry of 50 g of 230- to 400-mesh silica gel in 1:1 (v/v) hexanes/DCM. Precondition with the same solvent.

19. Dissolve the crude material in a minimum amount of 1:1 hexanes/DCM and load onto the column. Perform chromatography using a small amount of air pressure (APPENDIX 3E) and eluting with solvent mixtures of increasing polarity:

    1:1 (v/v) hexanes/DCM
    25:75 (v/v) hexanes/DCM
    DCM
    99:1, 98:2, 97:3, 96:4, and 95:5 (v/v) DCM/MeOH.

20. Combine the product-containing fractions into a 500-mL round-bottom flask and concentrate to a white solid (60% yield) on a rotary evaporator.

21. Determine the melting temperature. Characterize by TLC, ^{1}H and ^{13}C NMR, IR, and mass spectrometry.

    Boc-Arg(Fmoc)$_2$-OH: R_f 0.54 (93:5:2 DCM/MeOH/AcOH); m.p. 106°-109°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81-7.26 (m, 16H), 5.14 (m, 1H), 4.75 (m, 2H), 4.30 (m, 5H), 3.52 (m, 2H), 1.43 (s, 9H), 1.26 (m, 4H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 161, 159, 157, 128, 120, 78, 68, 47, 44, 29, 28, 25 ppm; IR (film): 3800, 1720, 1620, 1515, 1460, 1260, 1110 cm^{-1}; FAB-MS (positive mode, magic bullet, 3:1 dithiothreitol/dithioerythritol): m/z 719.4 [(Boc-Arg(Fmoc)$_2$-OH)+H]$^+$, calcd. mass for $C_{41}H_{42}N_4O_8$: 718.8.

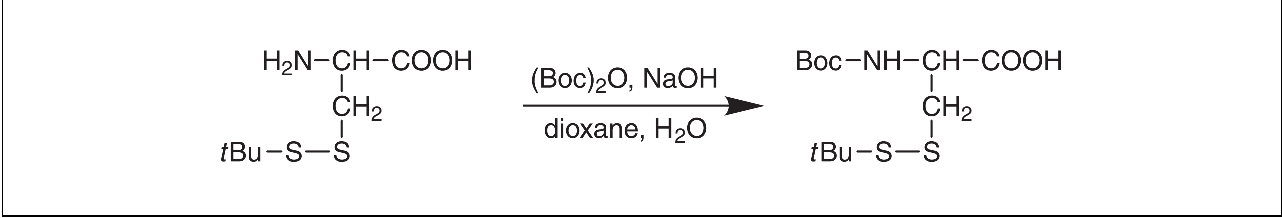

Figure 4.22.6 Protection of the α-amine of H-Cys(S-*t*Bu)-OH. Boc, *tert*-butoxycarbonyl.

PREPARATION OF Boc-Cys(S-*t*Bu)-OH

SUPPORT PROTOCOL 3

Boc-protection of cysteine is shown in Figure 4.22.6.

Materials

S-(*tert*-Butylthio)-L-cysteine (H-Cys(S-*t*Bu)-OH; Novabiochem)
2:1 (v/v) dioxane/water
1 M NaOH
Di-*tert*-butyldicarbonate (Boc_2O)
Hexanes
1 M HCl
Ethyl acetate (EtOAc)
Sodium sulfate (Na_2SO_4), anhydrous

50-mL and 250-mL round-bottom flasks
100-mL separatory funnel
Gravity filtration device and filter paper
Rotary evaporator equipped with water aspirator

Additional reagents and equipment for TLC (*APPENDIX 3D*), ^{1}H NMR, and mass spectrometry

Introduce Boc protecting group

1. Place 0.5 g (2.4 mmol) *S*-(*tert*-butylthio)-L-cysteine in a 50-mL round-bottom flask.
2. Suspend in 15 mL of 2:1 (v/v) dioxane/water and adjust the pH to 9 by addition of 1 M NaOH.
3. Add 0.60 g (2.74 mmol) di-*tert*-butyldicarbonate and keep the reaction overnight at room temperature with magnetic stirring.

   To avoid decomposition of di-tert-butyldicarbonate, store at –20°C.

Work up reaction

4. Transfer the reaction mixture to a 100-mL separatory funnel and wash twice with 50 mL hexanes.
5. Cool the aqueous phase in an ice bath and add 1 M HCl dropwise with manual stirring until the pH is 2 to 3.
6. Transfer the acidified aqueous phase to the 100-mL separatory funnel and extract three times with 50 mL EtOAc.
7. Wash the combined organic phases two times with 25 mL water.
8. Dry the organic layer over anhydrous Na_2SO_4. Add more Na_2SO_4 if the salt crystals clump together upon swirling.

9. Gravity filter the resulting solution through filter paper into a 250-mL round-bottom flask. Rinse the Na_2SO_4 crystals with 10 to 20 mL EtOAc.

10. Remove the solvent under reduced pressure (i.e., in a rotary evaporator with a water aspirator) to yield a white solid (80% yield). Use without further purification.

11. Determine the melting temperature. Characterize by TLC, ^{1}H NMR, and mass spectrometry.

Boc-Cys(S-tBu)-OH: R_f 0.47 (95:5 DCM/MeOH); m.p. 119°-120°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.40 (d, 1H, J = 7.8 Hz), 4.61 (m, 1H), 3.20 (m, 2H), 1.47 (s, 9H), 1.34 (s, 9H) ppm; FAB-MS (positive mode, 4-nitrobenzyl alcohol): m/z 309.4 [(Boc-Cys(S-tBu)-OH)+H]$^+$ and 332.4 [(Boc-Cys(S-tBu)OH)+Na]$^+$, calcd. mass for $C_{12}H_{23}NO_4S_2$: 309.5.

SUPPORT PROTOCOL 4

PREPARATION OF Boc-Hse(DMTr)-O$^-$HTEA$^+$

Boc protection and tritylation of homoserine are shown in Figure 4.22.7.

Materials

L-Homoserine (Novabiochem)
Pyridine, anhydrous (see recipe)
Argon (Ar), dry
Chlorotrimethylsilane
Triethylamine
Di-*tert*-butyldicarbonate (Boc_2O)
32% ammonium hydroxide
4,4′-Dimethoxytrityl chloride (DMTr·Cl)
Dichloromethane (DCM), neutralized (see recipe)
Methanol (MeOH)
Triethylamine
Acetonitrile, anhydrous (see recipe)

250-mL oven-dried round-bottom flask with rubber septum
Oven-dried glass syringes and needles
Rotary evaporator equipped with a water aspirator
500-mL round-bottom flask

Additional reagents and equipment for column chromatography (see Support Protocol 2 and *APPENDIX 3E*), TLC (*APPENDIX 3D*), ^{1}H and ^{13}C NMR, and mass spectrometry

Introduce Boc protecting group

1. Place 0.5 g (4.20 mmol) of L-homoserine in a 250-mL oven-dried round-bottom flask.

2. Dry by co-evaporating twice with 25 mL anhydrous pyridine.

3. Cap the flask with a rubber septum and purge the flask with dry Ar.

4. Add 10 mL anhydrous pyridine and 3.50 mL (25.2 mmol) chlorotrimethylsilane with oven-dried glass syringes and needles.

5. Let the reaction stand 2 hr under an Ar atmosphere at room temperature.

6. Add 1.1 g (5 mmol) di-*tert*-butyldicarbonate and 0.7 mL (5 mmol) triethylamine, and stir continuously 20 hr at room temperature under an Ar atmosphere.

7. Slowly add 10 mL cold water and 7 mL concentrated ammonium hydroxide (32% v/v), and stir 30 min at room temperature.

The final ammonium hydroxide concentration achieved (~ 2 M) will ensure complete removal of silyl groups.

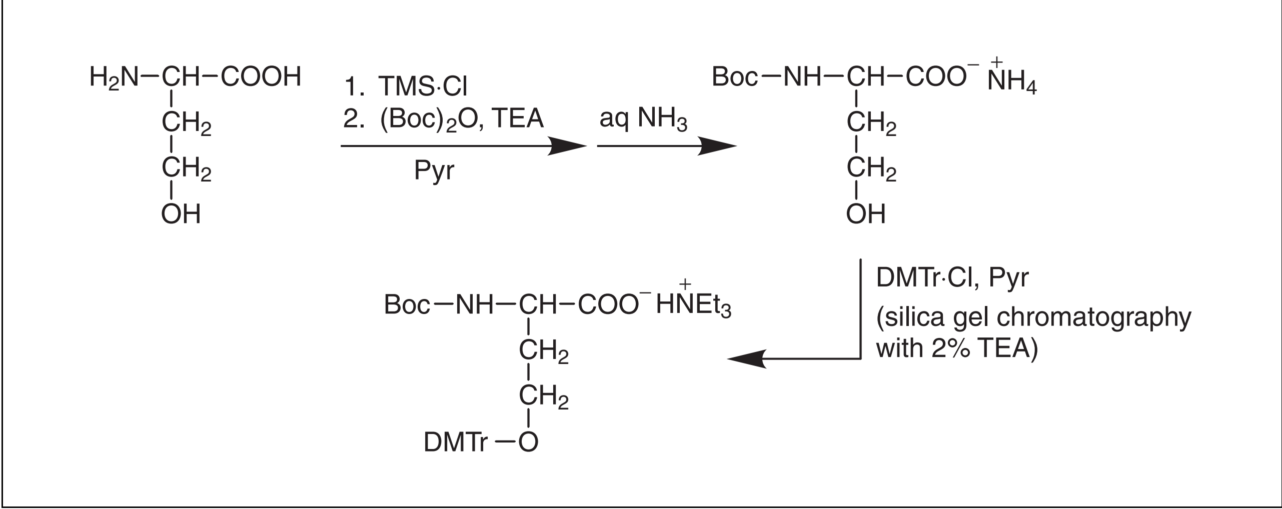

Figure 4.22.7 Synthesis of the homoserine derivative. Abbreviations: Boc, *tert*-butoxycarbonyl; DMTr, 4,4′-dimethoxytrityl; Pyr, pyridine; TEA, triethylamine; TMS, trimethylsilyl.

8. Evaporate to dryness under reduced pressure (i.e., in a rotary evaporator) to yield an oil.
9. Dry the remaining oil by co-evaporating twice with 25 mL anhydrous pyridine.
10. Dissolve in 10 mL anhydrous pyridine and add 1.75 g (5.2 mmol) DMTr·Cl.
11. Stir 24 hr at room temperature under an Ar atmosphere.
12. Evaporate to dryness under reduced pressure.

Isolate and characterize the product

13. Perform silica gel column chromatography (see Support Protocol 2, steps 18 to 19, and APPENDIX 3E), eluting with neutralized DCM and increasing amounts of MeOH (0% to 10%) in the presence of 2% triethylamine.
14. Combine the product-containing fractions in a 500-mL round-bottom flask and concentrate to an oil in a rotary evaporator.
15. Remove residual triethylamine by co-evaporating the oil with anhydrous acetonitrile to provide the pure product as a white solid (35% yield).
16. Determine the melting temperature. Characterize by TLC, ^{1}H and ^{13}C NMR, and mass spectrometry.

Boc-Hse(DMTr)-O^{-}HNEt$_3^{+}$: R_f 0.62 (9:1 DCM/MeOH); m.p. 67°-68°C; $[\alpha]_D$ = 9.93 (c 0.62, MeOH); ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.44-7.15 (m, 9H), 6.82-6.78 (m, 4H), 5.55 (d, 1H, J = 7.2 Hz), 4.22 (m, 1H), 3.77 (s, 6H), 3.30-3.10 (m, 2H), 2.98 (q, 6H, J = 7.3 Hz), 2.17 (m, 1H), 1.99 (m, 1H), 1.39 (s, 9H), 1.19 (t, 9H, J = 7.3 Hz) ppm; ^{13}C NMR (75 MHz, CDCl$_3$): δ 176.1, 158.3, 155.2, 145.1, 135.4, 130.0-126.5, 113.0, 86.1, 60.8, 55.1, 52.8, 45.0, 32.7, 28.3, 8.4 ppm; FAB-MS (positive mode, Xe, magic bullet, 3:1 dithiothreitol/dithioerythritol): m/z 623.1 [(Boc-Hse(DMTr)-O^{-}HNEt$_3^{+}$)+H]$^{+}$ and 522.1 [(Boc-Hse(DMTr)-OH)+H]$^{+}$, calcd. mass for $C_{38}H_{50}N_2O_7$ (Boc-Hse(DMTr)-O^{-}HNEt$_3^{+}$): 622.8, calcd. mass for $C_{30}H_{35}NO_7$ (Boc-Hse(DMTr)-OH): 521.6.

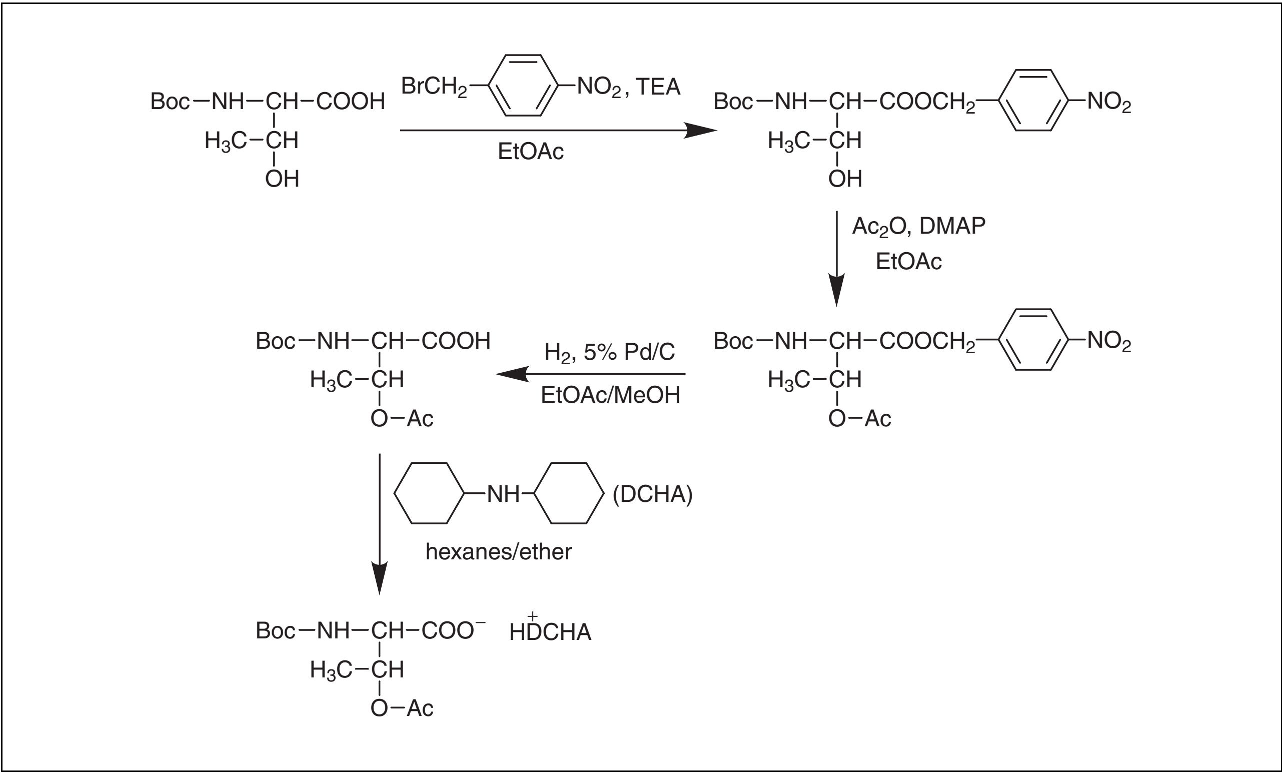

Figure 4.22.8 Protection of the threonine hydroxyl group and preparation of its solid dicyclohexylammonium salt. Abbreviations: Boc, *tert*-butoxycarbonyl; DCHA, *N,N*-dicyclohexylamine; DMAP, 4-(*N,N*-dimethylamino)pyridine; TEA, triethylamine.

SUPPORT PROTOCOL 5

PREPARATION OF Boc-Thr(Ac)-OH

Preparation of the protected threonine residue is shown in Figure 4.22.8.

Materials

N-(*tert*-Butoxycarbonyl)-L-threonine (Novabiochem)
Ethyl acetate (EtOAc)
4-Nitrobenzyl bromide
Triethylamine
1 M HCl
5% (w/v) aqueous sodium hydrogencarbonate ($NaHCO_3$) solution
Sodium sulfate (Na_2SO_4), anhydrous
Dichloromethane (DCM)
Methanol (MeOH)
Acetic anhydride
4-Dimethylaminopyridine (DMAP)
Argon (Ar), dry
5% (dry basis) palladium on activated carbon
Hydrogen gas
Hexanes
Diethyl ether
Dicyclohexylamine

100-, 250-, and 500-mL round-bottom flasks
Reflux condenser
Gravity filtration device and filter paper
250- and 500-mL separatory funnels
Rotary evaporator with a water aspirator
25-mL oven-dried round-bottom flask with rubber septum

Additional reagents and equipment for column chromatography (see Support Protocol 2 and *APPENDIX 3E*), TLC (*APPENDIX 3E*), ^{1}H and ^{13}C NMR, IR, and mass spectrometry

Prepare Boc-Thr-ONbn

1. Place 2.5 g (12.5 mmol) of *N*-(*tert*-butoxycarbonyl)-L-threonine in a 100-mL round-bottom flask. Add 20 mL EtOAc.
2. Add 3.4 g (15.7 mmol) 4-nitrobenzyl bromide and 1.6 g (15.7 mmol) triethylamine and reflux 6 hr.
3. Cool the resulting solution and gravity filter through filter paper.
4. Transfer the filtrate to a 500-mL separatory funnel and wash once with 50 mL water, once with 50 mL of 1 M HCl, and three times with 50 mL of 5% aqueous $NaHCO_3$ solution.
5. Dry the organic layer over anhydrous Na_2SO_4. Add more Na_2SO_4 if the salt crystals clump together upon swirling.
6. Gravity filter the resulting solution through filter paper into a 250-mL round-bottom flask. Rinse the Na_2SO_4 crystals with 10 to 20 mL EtOAc.
7. Remove the solvent under reduced pressure (i.e., in a rotary evaporator with a water aspirator).
8. Perform silica gel column chromatography (see Support Protocol 2, steps 18 and 19, and *APPENDIX 3E*), eluting with DCM and increasing amounts of MeOH (0% to 5%).
9. Combine product-containing fractions into a 500-mL round-bottom flask and concentrate to a white solid (70% yield) on a rotary evaporator.
10. Determine the melting temperature. Characterize by TLC (*APPENDIX 3D*), ^{1}H and ^{13}C NMR, IR, and mass spectrometry.

    Boc-Thr-ONbn: R_f 0.60 (95:5 $CHCl_3$/MeOH); m.p. 95°-98°C; $[\alpha]_D$ = –9.8 (c 0.62, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.2 (d, 2H, J = 8.8 Hz), 7.5 (d, 2H, J = 8.8 Hz), 5.4 (m, 1H), 5.3 (d, 1H, J = 3.2 Hz), 4.4 (m, 2H), 1.5 (s, 9H), 1.3 (d, 3H, J = 6.6 Hz) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 171, 156, 152, 143, 128, 124, 80, 68, 66, 59, 28, 20 ppm; IR (KBr): 3450, 1760, 1670, 1520, 1350 cm^{-1}; FAB-MS (positive mode, Xe, magic bullet): m/z 355.1 $[(Boc\text{-}Thr\text{-}ONbn)+H]^+$, 377.0 $[(Boc\text{-}Thr\text{-}ONbn)+Na]^+$, calcd. mass for $C_{16}H_{22}N_2O_7$: 354.36.

Prepare Boc-Thr(Ac)-ONbn

11. Place 3.2 g (9 mmol) of Boc-Thr-ONbn in a 100-mL round-bottom flask. Dissolve in 20 mL EtOAc.
12. Add 4.3 mL (45 mmol) acetic anhydride and 0.55 g (4.5 mmol) DMAP and stir for 2 hr at room temperature.
13. Add 10 mL MeOH and stir 10 min.
14. Concentrate to an oil in a rotary evaporator.
15. Perform silica gel column chromatography as in step 8.
16. Combine the product-containing fractions in a 500-mL round-bottom flask and concentrate to an oil (95% yield) in a rotary evaporator.

17. Characterize by TLC, ^{1}H and ^{13}C NMR, IR, and mass spectrometry.

Boc-Thr(Ac)-ONbn: R_f 0.84 (95:5 $CHCl_3$/MeOH); $[\alpha]_D = -7.2$ (c 0.23, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.2 (d, 2H, J = 8.8 Hz), 7.5 (d, 2H, J = 8.8 Hz), 5.4 (m, 1H), 5.2 (d,1H, J = 3.6 Hz), 4.5 (dd, 1H, J = 2.2, 9.4 Hz), 2.1 (s, 3H), 1.5 (s, 9H), 1.3 (d, 3H, J = 6.2 Hz), ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 177, 170, 156, 148, 142, 129, 124, 81, 70, 66, 57, 28, 21 ppm; IR (film): 1750, 1710, 1610, 1530, 1350 cm^{-1}; FAB-MS (positive mode, Xe, nitrobenzyl alcohol): m/z 397.0 [(Boc-Thr(Ac)-ONbn)+H]$^{+}$, calcd. mass for $C_{18}H_{24}N_2O_8$: 396.40.

Prepare Boc-Thr(Ac)-OH (oil)

18. Place 0.95 g (2.4 mmol) Boc-Thr(Ac)-ONbn into a 25-mL oven-dried round-bottom flask. Cap the flask with a rubber septum and purge with dry Ar.

19. Dissolve in 10 mL of 1:1 (v/v) EtOAc/MeOH.

20. Add 0.18 mg of 5% palladium on activated carbon. Bubble hydrogen gas into the reaction mixture for 3 hr.

    CAUTION: *Hydrogen is an extremely flammable gas. Keep the container in a well-ventilated place away from sources of ignition. Take precautionary measures against static discharges. Always manipulate in a well-ventilated fume hood and wear appropriate protective laboratory equipment.*

21. Eliminate the catalyst by gravity filtration through filter paper. Rinse with 10 to 20 mL MeOH.

22. Concentrate the filtrate to dryness in a rotary evaporator.

23. Dissolve the resulting oil in 100 mL EtOAc and transfer to a 250-mL separatory funnel.

24. Wash the organic layer six times with 50 mL of 1 M HCl and then three times with 50 mL water.

25. Dry the organic layer over anhydrous Na_2SO_4. Add more Na_2SO_4 if the salt crystals clump together upon swirling.

26. Gravity filter the resulting solution through filter paper into a 250-mL round-bottom flask. Rinse the Na_2SO_4 crystals with 10 to 20 mL EtOAc.

27. Remove the solvent under reduced pressure (i.e., in a rotary evaporator).

28. Perform silica gel column chromatography as in step 8.

29. Combine the product-containing fractions into a 500-mL round-bottom flask and concentrate to an oil (55% yield) in a rotary evaporator.

30. Characterize by TLC, ^{1}H and ^{13}C NMR, and IR.

Boc-Thr(Ac)-OH: R_f 0.71 (95:5 $CHCl_3$/MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.75 (m, 1H), 4.39 (m, 1H), 4.26 (d, 1H, J = 8.6 Hz), 4.15 (m, 1H), 1.45 (s, 9H), 1.25 (d, 3H, J = 5.6 Hz) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): 174, 170, 156, 81, 71, 57, 28, 21, 17 ppm; IR (film): 3400, 1750, 1710, 1520 cm^{-1}.

Prepare Boc-Thr(Ac)-O^{-}DCHA^{+} (solid)

31. Dissolve Boc-Thr(Ac)-OH in 1:1 (v/v) hexanes/diethyl ether and add an equimolar amount of dicyclohexylamine. Stir overnight at 0°C.

32. Filter and then wash with prechilled 1:1 (v/v) hexanes/diethyl ether to obtain a white solid (93% yield).

33. Characterize by TLC, ^{13}C NMR, and mass spectrometry.

Boc-Thr(Ac)-O^-DCHA^+: R_f 0.47 (85:10:5 $CHCl_3$/MeOH/AcOH); m.p. 172°-173°C; ^{13}C NMR (75 MHz, $CDCl_3$): δ 174, 170, 156, 78, 73, 59, 53, 29, 28, 25, 24, 21, 17 ppm; FAB-MS (positive mode, Xe, magic bullet, 3:1 dithiothreitol/dithioerythritol): m/z 443.0 [(Boc-Thr(Ac)-O^-DCHA^+)+H]$^+$ and 465.0 [(Boc-Thr(Ac)-O^-DCHA^+)+Na]$^+$, calcd. mass for $C_{23}H_{42}N_2O_6$: 442.60.

ELONGATION OF THE OLIGONUCLEOTIDE CHAIN AND CLEAVAGE AND DEPROTECTION OF THE NUCLEOPEPTIDE

BASIC PROTOCOL 2

In this protocol, the peptide-bound support is added to an oligonucleotide synthesis column and the nucleotide is extended from the appropriate amino acid hydroxyl function (homoserine in Fig. 4.22.2B). In general, oligonucleotide synthesis follows standard automated DNA methods (see *APPENDIX 3C*); however, some minor modifications have to be introduced in the oligonucleotide synthesis cycle, since a polystyrene support is used instead of controlled-pore glass (CPG). The main washes are made with neutralized DCM rather than acetonitrile, and THF washes are used after certain steps. A 2% *N*,*N*-diisopropylethylamine solution is used to wash the support after detritylation. Washes with anhydrous acetonitrile are employed before phosphoramidite couplings. The synthesis cycle is outlined in Table 4.22.3. Oligonucleotide synthesis is monitored by the trityl assay, and the yield of the first incorporation is also determined.

Complete deprotection of the nucleopeptide can be carried out using ammonium hydroxide alone or using tetra-*n*-butylammonium fluoride (TBAF) followed by ammonium hydroxide. Ammonia treatment may give (especially with glycine at the C terminus) a mixture of the C-terminal carboxylic acid and carboxamide nucleopeptides. The TBAF procedure gives exclusively the C-terminal carboxylic acid nucleopeptide irrespective of the bifunctional spacer used. The TBAF treatment is recommended if nucleopeptides are sufficiently stable, but may not be compatible with nucleopeptides in which serine or threonine are the linking residues (see Strategic Planning). In some cases, presence of tetra-*n*-butylammonium counterions renders the reversed-phase HPLC analysis (see Basic Protocol 3) difficult. In those cases, it is recommended to obtain the sodium salt of the nucleopeptide as described below. PAGE analysis (see Basic Protocol 4) is also more reliable with desalted products. For additional requirements for deprotection, analysis, and purification, see Support Protocol 7.

Materials

Peptide-derivatized support (see Basic Protocol 1)
DNA phosphoramidites (Glen Research): 5′-*O*-(4,4′-dimethoxytrityl)-*N*-protected-2′-deoxyribonucleoside-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl)-phosphoramidites, where the nucleobases are:
- N^6-benzoyladenin-9-yl
- N^2-isobutyrylguanin-9-yl
- N^2-dimethylaminomethyleneguanin-9-yl
- N^4-benzoylcytosin-1-yl
- N^4-acetylcytosin-1-yl
- thymin-1-yl

Argon (Ar) and nitrogen (N_2, optional) gas, dry
Anhydrous acetonitrile (see recipe) in a septum-sealed distillation collection bulb
Activator solution (see recipe)
Cap A and B capping reagents (Glen Research)
Oxidizer solution (see recipe)
Peroxide-free THF (see recipe)

Table 4.22.3 Automated 5-µmol Synthesis Cycle for the Oligonucleotide Chain Elongation on an ABI 380B DNA Synthesizer

Synthesis step	Function	Time (sec)
Detritylation		
1	DCM to waste	5
2	DCM to column	20
3	Ar reverse flush	10
4	Ar block flush	5
5	Waste port	1
6	Advance fraction collector	1
7	3% TCA to waste	5
8	3% TCA to column	60
9	Ar column flush	10
10	3% TCA to column	60
11	Ar column flush	10
12	DCM to waste	3
13	DCM to column	180
14	Ar column flush	10
15	Ar block flush	5
16	Waste to bottle	1
17	2% DIPEA to waste	3
18	2% DIPEA to column	30
19	Ar reverse flush	10
20	DCM to waste	3
21	DCM to column	20
22	Ar reverse flush	10
Column washing		
23	ACN to waste	5
24	ACN to column	45
25	Ar reverse flush	30
26	Ar block flush	5
27	ACN to waste	5
28	ACN to column	45
29	Ar reverse flush	45
30	Ar block flush	5
Phosphoramidite coupling		
30	Phosphoramidite preparation	3
31	Activator to column	3
32	Phosphoramidite + activator to column	25
33	Activator to column	3
34	THF to waste	5
35	Wait	300
36	THF to column	30
37	Ar reverse flush	10
38	Ar block flush	5

continued

Table 4.22.3 Automated 5-μmol Synthesis Cycle for the Oligonucleotide Chain Elongation on an ABI 380B DNA Synthesizer, *continued*

Synthesis step	Function	Time (sec)
Column capping		
39	Capping preparation	10
40	Cap A + cap B to column	30
41	Wait	120
42	DCM to waste	5
43	DCM to column	45
44	Ar reverse flush	10
45	Ar block flush	5
Oxidation and column washing		
46	Oxidizer to waste	7
47	Oxidizer to column	30
48	DCM to waste	5
49	Wait	60
50	THF to waste	5
51	THF to column	60
52	Ar reverse flush	10
53	DCM to waste	5
54	DCM to column	30
55	Ar reverse flush	10
56	Ar block flush	5
57	Repeat steps 54 and 55 three times	
58	Ar block flush	5

Detritylation solution: 3% (w/v) trichloroacetic acid (TCA, 99%, Glen Research) in neutralized DCM
2% (v/v) *N,N*-diisopropylethylamine (DIPEA) in neutralized DCM
Neutralized DCM (see recipe)
0.1 M *p*-toluenesulfonic acid monohydrate in acetonitrile
70% (v/v) perchloric acid
Absolute ethanol (EtOH)
Peroxide-free dioxane (see recipe)
32% ammonium hydroxide (store at 4°C)
1 M dithiothreitol (DTT) in peroxide-free dioxane
Tetra-*n*-butylammonium fluoride (TBAF, Aldrich)
Anhydrous tetrahydrafuran (see recipe) in a septum-sealed distillation collection bulb
Methanol (MeOH), HPLC grade
Glacial acetic acid
200- to 400-mesh Dowex 50WX4-400 ion-exchange resin (4.8 meq Na^+/g; Fluka Chemic)
1 M NaOH

Synthesis column for 5-μmol-scale synthesis: old empty OPC cartridge (ABI) with a body and two caps (13-mm aluminum seals; Chromatographic Specialties)

Empty DNA synthesizer bottles, oven dried, with rubber septa
Vacuum desiccator containing P_2O_5
Glass syringes and needles, oven dried
ABI 380B automated DNA synthesizer
External fraction collector and 15-mL test tubes
Double-beam UV spectrophotometer, calibrated, and quartz cuvettes
20-mL screw-cap pressure tubes, O-ring seal preferred
55°C oven
Disposable polypropylene syringes and polyethylene filters (cotton optional in some steps)
50-mL round-bottom flasks
Teflon two-way stopcocks
Rotary evaporator with a water aspirator
Lyophilizer
Vacuum filtration system

Additional reagents and equipment for automated oligonucleotide synthesis (*APPENDIX 3C*) and amino acid analysis (see Basic Protocol 1)

CAUTION: All solutions and reagents required for the DNA synthesizer should be manipulated and prepared in a well-ventilated fume hood.

NOTE: Oxidizer solution, DIPEA solution, neutralized DCM, and peroxide-free THF should be filtered (Pro-X[R] nylon filters, 0.25 µm, 25 mm) before they are added to the synthesizer bottles.

Prepare column

1. Transfer an accurately weighed amount of peptide-derivatized support for a 5-µmol synthesis (i.e., 100 mg peptide-support with a loading of 0.20 µmol/mg) to an assembled oligonucleotide synthesis column.

   The peptide-resin contains the hydroxyl function where the first nucleotide will be anchored and the oligonucleotide chain subsequently elongated.

   Standard 1-µmol scale columns may fit enough a resin for a 1 to 2-µmol-scale synthesis on polystyrene.

Perform oligonucleotide synthesis

2. Weigh the appropriate amount of DNA phosphoramidites in oven-dried synthesizer bottles, cap with rubber septa, insert a disposable needle, and dry them in a P_2O_5-containing desiccator under vacuum (6 hr minimum).

3. Open the desiccator under an Ar atmosphere. Using oven-dried glass syringes and needles, dissolve the compounds in sufficient anhydrous acetonitrile (from a septum-sealed distillation collection bulb) to yield a 0.2 M concentration.

   Care must be taken to avoid the presence of moisture throughout the entire process.

4. Place the synthesizer reagents in the following ports of an ABI 380B synthesizer:

   Activator: port 9
   Cap A and B: ports 11 and 12
   Oxidizer: port 15
   Peroxide-free THF: port 16
   Detritylation solution: port 14
   2% DIPEA in neutralized DCM: port 17
   Neutralized DCM: port 18
   Anhydrous acetonitrile: port 13
   DNA phosphoramidite solutions (step 3): ports 1 to 4.

5. Enter the sequence to be synthesized. Introduce an additional nucleotide at the 3′ end that will correspond to the peptide-resin containing the hydroxyl function where the oligonucleotide will be anchored.

   For example, to synthesize the tryptophan-containing nucleopeptide shown in Figure 4.22.2B, enter the sequence 5′-GCTACGT-3′, where the 3′-T corresponds to the peptide-linked resin.

6. Perform synthesis in the DMTr-off mode using a synthesis cycle modified as outlined in Table 4.22.3. Collect DMTr cation solutions in 15-mL test tubes using an external fraction collector.

   When nucleopeptides with >10 nucleobases are prepared, synthesis in DMTr-on mode is recommended in order to facilitate the purification.

7. Upon completion of the synthesis, dry the resin by manually conducting an Ar reverse flush operation on the synthesizer for 2 to 3 min. Alternatively, dry the support under a stream of N_2 or Ar.

Monitor DNA synthesis by DMTr assay

8. Dilute the first three and last three DMTr cation solutions to 10 mL with 0.1 M *p*-toluenesulfonic acid monohydrate in acetonitrile. Mix thoroughly.

   CAUTION: *Handle the solution containing trichloroacetic acid in DCM and acetonitrile with gloves, because it is corrosive as well as toxic. Always manipulate in a well-ventilated fume hood.*

   The volume of DMTr solutions may change during the course of a synthesis because DCM is a volatile solvent. Fractions may sit for several days before being assayed without affecting the results. Samples that have gone to dryness must be thoroughly redissolved. Addition of the p-toluenesulfonic acid ensures ionization of the DMTr group, making the solutions more strongly colored.

9. Dilute each sample 20- to 50-fold with the same solution. Measure the absorbance at 498 nm (A_{498}) versus the 0.1 M *p*-toluenesulfonic acid monohydrate solution in acetonitrile.

   These dilutions are necessary because high absorbance measurements are not reliable.

10. Calculate the rough stepwise coupling efficiency for the synthesis as a whole, using the following equation:

$$(\text{stepwise efficiency})^n = \frac{\text{avg. absorbance last three fractions}}{\text{avg. absorbance first three fractions}}$$

Equation 4.22.2

   where n is the number of DMTr nucleotides in the oligonucleotide (equal to the length of the oligonucleotide for DMTr-off synthesis).

Quantify incorporation yield of the first nucleotide

11. Evaporate the first DMTr cation solution to dryness and redissolve the remaining residue in a known volume of 3:2 (v/v) 70% perchloric acid/EtOH.

    CAUTION: *Handle 3:2 (v/v) 70% perchloric acid/EtOH with gloves in a well-ventilated fume hood. Perchloric acid is very corrosive and can be explosive when it contacts organic materials. Perchloric-containing residues must be appropriately neutralized prior to disposal.*

12. Dilute the sample 20- to 50-fold with the same solution in order to obtain an A_{498} <1. Measure the A_{498} of the sample versus the 3:2 (v/v) 70% perchloric acid/EtOH solution.

13. Calculate loading as:

$$\text{loading} = \frac{A_{498} \times \text{DF} \times 1000}{\varepsilon \times l \times m}$$

Equation 4.22.3

where DF is the dilution factor, ε is the molar extinction coefficient of the DMTr cation at 498 nm (71,700 L/mol·cm), l is the absorbance path of the cuvette (in cm), and m is the amount of peptide-support introduced in the column (in mg).

14. Determine the incorporation yield of the first nucleotide onto the peptide-support as the ratio between the obtained loading value and the peptide-support loading determined by amino acid analysis.

Perform complete deprotection of nucleopeptide

With ammonium hydroxide treatment

15a. Open the column and transfer the nucleopeptide-support to a 20-mL screw-cap pressure tube, preferably with an O-ring seal.

16a. Add 5 mL peroxide-free dioxane and 5 mL of 32% ammonium hydroxide. Screw the cap on tightly, and incubate 15 hr at 55°C in an oven or 6 to 8 hr at room temperature (see Strategic Planning for more details). When the nucleopeptide contains Cys(S-*t*Bu), use 5 mL of 1 M DTT in dioxane and 5 mL of 32% ammonium hydroxide and treat for 6 to 8 hr at room temperature (see Support Protocol 7).

CAUTION: *The 32% ammonium hydroxide solution must be kept at 4°C with the cap tightly sealed. Concentrated ammonium hydroxide is extremely caustic. Breathing the vapors is harmful. Always wear glasses and gloves when using this compound and work in a well-ventilated fume hood, as it is possible to be quickly overcome by ammonia fumes and be blinded.*

17a. Cool the mixture to room temperature. Filter through a disposable polypropylene syringe with a polyethylene filter or cotton, and collect in a 50-mL round-bottom flask.

18a. Wash the pressure tube several times with MeOH and pass the washes through the syringe. Concentrate the resulting filtrate under reduced pressure to a volume of ~2 mL.

To prevent inadvertent detritylation of dimethoxytritylated nucleopeptides, avoid heat and acid during the evaporation step. Elimination of dioxane and ammonium hydroxide should be done at room temperature to preserve the 5′-DMTr on the oligonucleotide chain. Addition of triethylamine is recommended to ensure no loss of the DMTr group.

With TBAF and ammonium hydroxide treatment

15b. Open the column and transfer the nucleopeptide-support into a polypropylene disposable syringe with a polyethylene filter closed with a stopcock.

16b. Add 5 mL of 0.05 M TBAF in anhydrous THF and let stand 30 min, stirring occasionally.

17b. Collect the filtrate in a 50-mL round-bottom flask. Wash three times with 2 mL MeOH followed by 5 mL THF and add to the filtrate. Add a few drops of glacial acetic acid and evaporate to dryness using a rotary evaporator.

18b. Redissolve the residue in 10 mL of 1:1 (v/v) peroxide-free dioxane/32% ammonium hydroxide and place in a 55°C oven for 15 hr, or at room temperature for 6 to 8 hr, depending on the stability expected for the target nucleopeptides. Concentrate the resulting filtrate under reduced pressure to a volume of ~2 mL.

Obtain sodium salt of nucleopeptide (optional; for TBAF only)

19. Add 200- to 400-mesh Dowex 50WX4-400 ion-exchange resin to a polypropylene syringe fitted with a polyethylene disc.
20. Wash with 200 mL water.
21. Wash with 200 mL of 1 M NaOH, added dropwise.
22. Wash with water until the solution has the pH of deionized water.
23. Dissolve deprotected nucleopeptide residue (step 18b) in a minimum amount of water (1 to 2 mL) and load onto the equilibrated Dowex resin. Elute with water until no UV-absorbing filtrate is obtained (check by measuring the absorbance at 260 nm versus water). Collect 1 to 2 mL fractions in disposable tubes.
24. Combine the product-containing fractions and lyophilize.

Calculate cleavage yield

25. Wash the resin (resulting from ammonia or TBAF treatment; step 17a or 17b) with MeOH and dry directly on a vacuum filtration system.
26. Proceed with amino acid analysis as described (see Basic Protocol 1, steps 9 to 13).
27. Calculate the cleavage yield as the ratio between any amino acid in the nucleopeptide still attached to the solid support and the internal reference amino acid.

    Choose residues that are not destroyed or modified during the hydrolysis process, such as amino acids with aliphatic side chains, phenylalanine, or aspartic and glutamic acids.

ANALYSIS, PURIFICATION, AND CHARACTERIZATION OF NUCLEOPEPTIDES

BASIC PROTOCOL 3

Crude nucleopeptides are first analyzed and then purified by liquid chromatography techniques. Analysis is routinely carried out using reversed-phase HPLC (RP-HPLC), although analytical PAGE (see Basic Protocol 4) can provide additional information, or may be required if the sample is highly impure. Purification is also generally performed by RP-HPLC. Medium-pressure systems (MPLC) may be the best option for relatively homogeneous crude nucleopeptides, since relatively large amounts of product (30 to 1000 OD units) can be purified in a single run. When this technique is inefficient, or if complicated crude nucleopeptides are obtained, the higher resolving power of high-pressure systems is required. Either analytical or semipreparative HPLC columns can be used, depending on the amount of pure product to be isolated. Preparative gel electrophoresis (see Basic Protocol 4) provides very good resolution and may allow pure nucleopeptides to be obtained when RP-HPLC fails. Its main disadvantage is low recovery of product from the gel.

After purification, the overall synthesis yield is determined and the nucleopeptide is quantified by spectrophotometry at 260 nm. The nucleopeptide is then analyzed by MALDI-TOF or ESI mass spectrometry. Oligonucleotide analysis, purification, and characterization procedures are described in *UNITS 10.1-10.7*. Base composition is determined by digestion using a combination of alkaline phosphatase (AP) and either snake venom phosphodiesterase (SVPD) or calf spleen phosphodiesterase (SpPD). The assessment of base composition is more often carried out using SVPD than SpPD. Degradation with SVPD and AP can be performed in a single reaction mixture, because both enzymes work at the same slightly basic pH. Degradation with SpPD and AP requires two separate

treatments under different conditions. The same information is generally obtained using either protocol, and consistent results from both reinforce that the nucleoside composition is as expected.

Finally, the product is analyzed by amino acid analysis after acid hydrolysis or enzymatic digestion of the peptide moiety. Enzymatic digestion is carried out in a sequential mode using three enzymes: papaine, microsomal leucin-aminopeptidase, and prolidase. (1) Papaine is a nonspecific enzyme that cleaves at -X-↑-Y-, where Y is not specific and X can be any residue, but is preferably arginine, lysine, or residues consecutive to phenylalanine. (2) Microsomal leucin-aminopeptidase is an exopeptidase that hydrolyzes peptide bonds adjacent to a free α-amine group (H_2N-X-↑-Y-, where X is not proline and Y is not specific). It does not hydrolyze γ-amino groups. (3) Prolidase is a dipeptidase that hydrolyzes dipeptides with a proline or hydroxyproline residue at the C terminus (H_2N-X-↑-Y-, where X is not proline, but Y is proline or hydroxyproline). When coupled with amino acid analysis, this protocol allows the content of asparagine and aspartic acid to be determined, as well as other acid-labile amino acids (e.g., methionine, tryptophan).

Materials

Crude deprotected nucleopeptide (see Basic Protocol 2 or Support Protocol 7)
0.01 M ammonium acetate, pH 7.0
HPLC mobile phase A: 0.01 M ammonium acetate, pH 7.0
HPLC mobile phase B: 1:1 (v/v) acetonitrile/water
Methanol (MeOH), HPLC grade
MPLC mobile phase A: 0.05 M ammonium acetate, pH 7.0
MPLC mobile phase B: 70% (v/v) 0.05 M ammonium acetate solution, pH 7.0, in 1:1 (v/v) acetonitrile/water
Matrix: 2′,4′,6′-trihydroxyacetophenone (THAP) or 3-hydroxypicolinic acid (3-HPA)
Ammonium citrate
Acetonitrile, HPLC grade
0.1% (v/v) triethylamine in water
Isopropanol (optional)
0.1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.1 M $MgCl_2$
1.5 U/500 µL snake venom phosphodiesterase (SVDP; Boehringer-Mannheim; EC 3.1.4.1)
0.0337 U/µL bacterial alkaline phosphatase (AP; Sigma; EC 3.1.16.1)
0.2 M ammonium acetate, pH 5.4 and 8.3
0.23 U/µL calf spleen phosphodiesterase (SpPD; Sigma; EC 3.1.3.1)
Concentrated HCl
0.1 M ammonium acetate, pH 5.3
1:32 (v/v) 2-mercaptoethanol/water
20 mg/mL (286 U/mL) papaine (from *Papaya latex*; Sigma; EC 3.4.22.2)
Glacial acetic acid
3 mg/mL (72 U/mL) microsomal leucin aminopeptidase (mLAP type VI-S from kidney pork microsomes; Sigma; EC 3.4.11.2)
0.025 M $MnCl_2$ buffer solution, pH 8.3
5 mg/mL (875 U/mL) prolidase (from pork kidney; Sigma; EC 3.4.13.9)

High-performance liquid chromatograph (HPLC) with:
Injector (autosampler preferred), sample loop, and syringe (for manual loading)
0.1 to 5 mL/min pumping system (binary)
UV/Vis detector, variable wavelength between 190 and 600 nm (preferred) or dual-wavelength detection

Data integrating system
Gradient system: displays and stores for redisplay and reformatting (preferred) or programmable
Analytical column: reversed-phase C18 column (i.e., Kromasil, 10 µm, 4.0 × 250 mm)
Semipreparative column: reversed-phase C18 column (i.e., Kromasil, 10 µm, 10.0 × 250 mm)
Automatic fraction collector (optional)
Lyophilizer
Double-beam UV spectrophotometer, calibrated
Quartz cuvettes
Preparative chromatographic system (medium-pressure liquid chromatography, MPLC):
Piston pump
Reversed-phase C18-filled glass column (i.e., Vydac 15 to 20-µm i.d., 300-Å porosity, 22 × 2 cm)
Automatic fraction collector
UV/Vis detector with fixed-wavelength detection
Teflon tubing connectors and adapters
Gradient-forming device with two equal-diameter cylinders and a Teflon stopcock between them
Chart recorder
Pyrex tubes
37°C water bath or heating block
Benchtop centrifuge

Additional reagents and equipment for reduction of sulfoxide-protected methionine (see Support Protocol 6; optional), deprotection of *tert*-butylthio-protected cysteine (see Support Protocol 7; optional), MALDI-TOF-MS (*UNIT 10.1*), ESI-MS (*UNIT 10.2*), and amino acid analysis (see Basic Protocol 1)

Analyze crude nucleopeptide by analytical RP-HPLC

1. Take 0.05 to 0.10 OD units of crude deprotected nucleopeptide and dilute with 0.01 M ammonium acetate buffer, pH 7.0, to a volume of 25 to 100 µL.

   Typical injection volumes for analysis are 10 to 100 µL depending on the sample loop size of the injector.

   For nucleopeptides containing tert-butylthio-protected cysteine, perform deprotection (see Support Protocol 7) prior to the analysis of the crude product.

2. Program the HPLC gradient system to start with 5% HPLC mobile phase B, increasing the percentage of HPLC mobile phase B with time (Table 4.22.4). Ensure that there is sufficient mobile phase to keep intakes covered throughout the run.

   For 5′-dimethoxytritylated nucleopeptides 5% to 60% mobile phase B in 30 min is the gradient recommended. For post-HPLC detritylation, see UNIT 10.5.

3. Set the UV detector wavelength at 260 nm. Using an analytical column, equilibrate the HPLC system with the starting mobile phase composition until a flat baseline is achieved at the desired detection wavelength.

4. Inject the sample by programming the autosampler or by loading it into the sample loop using an appropriate syringe. Elute the sample by starting the gradient. Record the chromatogram.

Table 4.22.4 Gradient and Mobile Phase for RP-HPLC of Crude Nucleopeptide[a]

Elapsed time (min)	Percent mobile phase B[b]
0	5
30	35
31	100
35	100
36	5
45	5

[a]Gradient conditions are based on a flow of 1 mL/min using an analytical Kromasil reversed-phase C18 column (10-µm diameter spherical silica, 4.0 × 250 mm) at ambient temperature with a 45-min injection cycle. Mobile phase A: 0.01 M ammonium acetate, pH 7.0; mobile phase B: 1:1 (v/v) acetonitrile/water.
[b]Percentage is at elapsed time.

Typically, nucleopeptides will elute at ~12 to 18 min under the recommended conditions (Table 4.22.4). For 5′-dimethoxytritylated nucleopeptides, the elution time will be longer (~18 to 25 min).

In some cases, when the nucleopeptide is small (e.g., <8 nucleobases) and has been cleaved with ammonium hydroxide, it is possible to differentiate the nucleopeptides with C-terminal carboxylic acid and carboxamide groups.

When the nucleopeptide contains sulfoxide-protected methionine or proline, it is possible to obtain two or more peaks (depending on the number of these residues) due to sulfoxide diastereomers or proline conformations (cis or trans).

5. For nucleopeptides containing sulfoxide-protected methionine, proceed with reduction (see Support Protocol 6) prior to purification.

Purify crude nucleopeptide by analytical or semipreparative RP-HPLC

6a. *For small amounts of nucleopeptide (1 to 10 OD units):* Perform HPLC as in steps 1 to 4. Load the analytical column with ~1 to 2 OD units at every injection and elute at a flow rate of 1 mL/min. Collect the desired fractions either with an automated fraction collector or by observing the chromatogram in real time and manually collecting the eluate.

6b. *For larger amounts of nucleopeptide (>10 OD units):* Use a semipreparative column under the described conditions. Elute at a flow rate of 3 mL/min.

7. Lyophilize the collected solution and quantify the amount of product obtained (steps 8 to 11).

Quantify nucleopeptide

8. Dissolve the lyophilized nucleopeptide in 5 mL water. Take 20 µL and dilute to a volume of 1 mL with water.

   An adequate dilution factor has to be chosen to keep absorbance <1. Modify the dilution depending on the synthesis scale and expected yield.

9. Measure the absorbance of the diluted sample in a quartz cuvette at 260 nm (A_{260}) on a calibrated, double-beam, UV spectrophotometer using water as a reference.

   If using a single-beam spectrophotometer, a blank should be run with a sample containing water (blank).

Table 4.22.5 Molar Extinction Coefficients of Nucleobases

Nucleobase (n_i)	ε_i at 260 nm
T	8830
dC	7700
dG	11500
dA	15200

10. Determine the amount of product (in OD_{260} units) and the concentration of the stock solution using the dilution factor and Beer's Law ($A_{260} = \varepsilon lc$), where ε is the molar extinction coefficient (see below), l is the path length of the UV cell (typically 1 cm), and c is the molar concentration of oligonucleotide. Lyophilize the nucleopeptide solution.

    In a nucleopeptide, the contribution of aromatic amino acids (tryptophan, phenylalanine, and tyrosine) to the global oligonucleotide absorbance at 260 nm is considered negligible in comparison with the absorption of the nucleobases.

    The molar extinction coefficient of the nucleopeptide is given by $\varepsilon = \varepsilon_i \times F$, where ε_i represents the molar extinction coefficient of each nucleobase (see Table 4.22.5) and F is a correction factor (0.9 when the oligonucleotide is single-stranded and 0.8 when it is double-stranded or self-complementary).

11. Calculate the overall synthesis yield. From the ratio of product obtained (in OD units) to the theoretical amount (calculated from the loading of the C-terminal amino acid resin).

    Average yields for nucleopeptide synthesis, cleavage, and purification are ~10% to 30%.

Purify crude nucleopeptide by preparative MPLC

12. Prepare a preparative chromatographic system as follows:
    a. Connect a piston pump to the top of a preparative reversed-phase C18-filled glass column.
    b. Connect the bottom to an automatic fraction collector through a UV/Vis detector.
    c. Connect the pump to the mixing chamber of a gradient-forming device. Connect the detector to a chart recorder using the appropriate ports.
13. Wash the column with 100 mL MeOH and 200 mL water by placing the pump entrance line in the appropriate bottles.
14. Set the UV detector wavelength at 260 nm with the appropriate lamp and filter. Turn on the detector and chart recorder.
15. Equilibrate the column with 200 mL MPLC mobile phase A at 3 to 4 mL/min. Stop the pump.
16. Place 600 mL MPLC mobile phase A and 600 mL MPLC mobile phase B in the appropriate compartments of the gradient-forming device. Place a magnetic stir bar in the mixing chamber (initially MPLC mobile phase A) and a magnetic stirrer below.
17. Place the pump solvent line inside the mixing chamber.
18. Dissolve the crude nucleopeptide sample in MPLC mobile phase A to a volume of 5 mL.
19. Remove the supernatant solution from the column cap position. Introduce the nucleopeptide sample solution onto the column and close.

20. Turn on the pump (3 to 4 mL/min) and the magnetic stirrer. Open the stopcock of the gradient-forming device to generate the gradient. Turn on the automatic fraction collector.

    Preparative chromatographic elution profiles are very similar to analytical ones. The gradient conditions described above extrapolate the typical analytical gradient (from 5% to 35% HPLC mobile phase B in mobile phase A). When DMTr-on nucleopeptides are purified, MPLC mobile phase B should be changed to 40% 0.05 M ammonium acetate solution, pH 7.0, in 1:1 (v/v) acetonitrile/water.

21. Select the tubes containing the desired product. Reanalyze those solutions by analytical HPLC (steps 1 to 4).

    In general, the desired nucleopeptide (main chromatographic peak) is eluted within four or five tubes and has a high level of purity (>95% to 99% as assessed by analytical HPLC).

22. Pool the fractions with the same chromatographic profile and purity requirements. Lyophilize the collected solution and quantify (steps 8 to 11).

Analyze nucleopeptide by MALDI-TOF-MS

23. Weigh 10 mg THAP matrix or 50 mg 3-HPA matrix into a 1.5-mL microcentrifuge tube. In a separate tube, weigh 50 mg ammonium citrate.

    Optimal results are obtained when the highest-purity matrices are used. Matrix and ammonium citrate solutions should be made fresh prior to use. Storage for >3 days is not recommended.

    In general, MALDI-TOF analysis of nucleopeptides is carried out following the same protocols as for simple oligonucleotides (UNIT 10.1). The standard matrices are used (THAP and 3-HPA) and negative-ionization mode is recommended because of the global negative charge of the nucleopeptides. For routine nucleopeptide analysis (up to 8 to 10 nucleobases), THAP is recommended. 3-HPA should be used when THAP does not give positive results.

24. Add 1 mL of 1:1 (v/v) acetonitrile/water to the THAP or 3-HPA matrix, and 1 mL water to the ammonium citrate salt. Vortex for 30 sec to allow complete dissolution.

25. Prepare a 50 to 100 µM nucleopeptide solution in water.

26. To a 0.5-mL microcentrifuge tube, add 1 µL nucleopeptide sample solution, 1 µL ammonium citrate solution, and 1 µL matrix solution. Mix by withdrawing and expelling the solution ten times with a pipet.

27. Spot 1 µL on the MALDI-TOF sample plate and dry. To allow for homogeneous crystallization, do not disturb the spotted sample after it starts to crystallize.

    Do not load the sample plate into the mass spectrometer before the plate is dry. Good signal can be obtained from anywhere around the edges of the crystallized spot.

28. Analyze by MALDI-TOF-MS (see *UNIT 10.1*).

Analyze nucleopeptide by ESI-MS

29. Prepare a 100 to 200 µM stock nucleopeptide solution in water.

    The best concentration for carrying out ESI-MS analysis depends on the instrument used. In general, nucleopeptides are analyzed by ESI-MS in the negative mode, as with MALDI-TOF-MS. See UNIT 10.2 for more specific details.

30. Add 5 µL of 0.1% triethylamine to 100 µL nucleopeptide solution.

31. Mix 100 µL of the above solution with an equal volume of acetonitrile (preferred), isopropanol, or MeOH.

    In general, the aqueous nucleopeptide solution is mixed in a 1:1 (v/v) ratio with an organic solvent such as acetonitrile, MeOH, or isopropanol, because the solvent is readily evaporated and facilitates the transfer of the ion from the liquid to the gas phase. The solvent also allows for the generation of a large number of ions. Another issue is that, as the pH increases, more negative ions are produced and the signal intensity rises. Addition of an organic base such as triethylamine to the nucleopeptide solution increases the pH and also reduces the extent of cation adducts observed in the mass spectra.

32. Analyze by ESI-MS (*UNIT 10.2*).

Analyze base composition by SVPD/AP digestion

33. Evaporate 0.5 to 1.0 OD unit of nucleopeptide sample to dryness under vacuum in an appropriate vessel (i.e., 1.5-mL microcentrifuge tube).

34. Add the following:

    34 µL freshly deionized water
    50 µL 0.1 M Tris·Cl, pH 8.0
    10 µL 0.1 M $MgCl_2$
    1 µL 1.5 U/500 µL SVPD
    5 µL 0.0337 U/µL bacterial AP.

35. Vortex the sample and centrifuge briefly to collect the liquid at the bottom of the tube. Incubate 8 to 15 hr at 37°C.

36. Vortex the sample and chill with dry ice at least 10 min.

37. Centrifuge 5 min in a benchtop centrifuge at maximum speed, room temperature. Carefully remove the supernatant with a pipet and transfer to a new labeled tube. Discard the original tube containing the pellet.

38. Evaporate the sample to complete dryness under vacuum.

39. Dissolve the dried sample in 50 µL water and vortex at least 30 sec.

Table 4.22.6 Gradient and Mobile Phase for RP-HPLC Analysis of Nucleopeptide Digests[a]

Elapsed time (min)	Percent mobile phase B[b]
0	10
5	10
20	30
25	100
30	100
31	10
40	10

[a]Gradient conditions are based on a flow of 1 mL/min using a Kromasil reversed-phase C18 column (10-µm diameter spherical silica, 4.0 × 250 mm) at ambient temperature with a 40-min injection cycle. Mobile phase A: 0.01 M ammonium acetate, pH 7.0; mobile phase B: 1:1 (v/v) acetonitrile/water.
[b]Percentage is at elapsed time

40. Perform reversed-phase HPLC of the digested sample on a C18 column. Load ~0.2 OD unit per injection and elute with an acetonitrile/ammonium acetate/water gradient as specified in Table 4.22.6. Set the detector to 260 nm.

See UNIT 10.6 for more specific details on RP-HPLC.

41. Collect data for 30 min.

The nucleoside elution order depends on the column and HPLC system used. Approximate elution times are 5.0 min for dC, 6.0 min for dG, 6.7 min for T, and 13.1 min for dA.

42. To calculate the base composition, use the molar extinction coefficients from Table 4.22.5 and the integrated area of each peak. First calculate the average value of the ratio between the integrated area and the molar extinction coefficient (X):

$$X = \sum (\text{peak area}/\varepsilon_i) / N$$

Equation 4.22.4

where ε_i is the molar extinction coefficient of each nucleoside (Table 4.22.5) and N the number of every particular nucleobase in the sequence. Then calculate the composition for each nucleoside:

$$\text{n}_i = (\text{peak area}/\varepsilon_i) / X$$

Equation 4.22.5

where n_i represents the experimental calculated value for the nucleoside i.

Although SVPD is a 3′-exonuclease, it has been shown to degrade nucleopeptides where the 3′-hydroxyl function of the oligonucleotide chain is blocked by linkage to a peptide chain. This modification would prevent degradation during a short incubation time, but not after long treatments as described above. Also, SVPD contains some endonuclease contamination that favors complete degradation of the oligonucleotide chain.

In general, the nucleoside composition calculated after enzymatic digestion is very similar to the expected composition. In some cases, the value of the 3′-nucleoside is low depending on the amino acid that links the peptide and oligonucleotide chain. When homoserine serine, or threonine is used, an extra peak, with higher retention time than that of simple nucleosides, may be observed in the RP-HPLC profile that corresponds to the 3′-nucleoside-peptide.

Analyze base composition by SpPD/AP digestion

43. Evaporate 0.5 to 1.0 OD unit of nucleopeptide sample to dryness under vacuum in an appropriate vessel (i.e., 1.5-mL microcentrifuge tube).

44. Add 30 µL of 0.2 M ammonium acetate buffer, pH 5.4, and 10 µL of 0.23 U/µL SpPD.

45. Vortex the sample and centrifuge briefly to collect the liquid at the bottom of the tube. Incubate 12 to 15 hr at 37°C.

46. Lyophilize and then add the following:

    34 µL freshly deionized water
    50 µL 0.1 M Tris·Cl, pH 8.0
    10 µL 0.1 M $MgCl_2$
    5 µL 0.0337 U/µL bacterial AP.

47. Continue as in steps 35 to 42.

SpPD is a 5′-exonuclease enzyme, and degrades the oligonucleotide chain of the nucleopeptide starting from the 5′-terminal hydroxyl group. SpPD also degrades the 3′-nucleoside-peptide linkage, independently of the linking amino acid.

In general, nucleoside composition after enzymatic digestion is very similar to the expected composition. In some cases, however, the deoxyadenosine value is lower than expected. This is because of deaminase contamination, and depends on the enzyme batch employed. In these cases, an extra peak appears in the HPLC trace, which is attributed to a modified deoxyadenosine nucleoside.

Perform amino acid analysis of nucleopeptide under acidic conditions

48. Place 5 OD_{260} units of purified nucleopeptide in a Pyrex tube.

49. Add 300 µL water and 300 µL concentrated HCl.

50. Determine amino acid content as described (see Basic Protocol 1, steps 11 to 13).

When quantifying amino acids from peptide-oligonucleotide conjugates, high values of glycine are obtained due to co-elution with oligonucleotide hydrolysis side-products.

Perform enzymatic analysis of peptides and nucleopeptides

51. Evaporate 3 to 5 nmol peptide or nucleopeptide sample to dryness under vacuum in an appropriate vessel (i.e., a 1.5-mL microcentrifuge tube).

52. Dissolve the sample in 15 µL of 0.1 M ammonium acetate buffer, pH 5.3. Add 1 µL of 1:32 (v/v) 2-mercaptoethanol/water.

53. Add 3 µL of 20 mg/mL (286 U/mL) papaine solution and incubate 2 hr at 37°C.

54. Add one drop of glacial acetic acid and lyophilize.

55. Redissolve the sample in 15 µL of 0.2 M ammonium acetate buffer, pH 8.3, and 1 µL of 1:32 (v/v) 2-mercaptoethanol/water.

56. Add 3 µL of 3 mg/mL (72 U/mL) microsomal leucin aminopeptidase solution and incubate 3 hr at 37°C.

57. Repeat the addition of 3 µL enzyme solution and incubate another 12 hr at 37°C.

58. Add one drop of concentrated acetic acid and lyophilize.

59. Redissolve the sample in 15 µL of 0.1 M ammonium acetate and 0.025 M $MnCl_2$ buffer solution, pH 8.3.

60. Add 3 µL of 5 mg/mL (875 U/mL) prolidase solution and incubate 3 hr at 37°C.

61. Add one drop of concentrated acetic acid and lyophilize.

62. Perform amino acid analysis as described (see Basic Protocol 1, step 13).

REDUCTION OF SULFOXIDE-PROTECTED METHIONINE-CONTAINING NUCLEOPEPTIDES

SUPPORT PROTOCOL 6

Additional Materials *(also see Basic Protocol 3)*

Deprotected crude nucleopeptide (see Basic Protocol 2)
N-Methylmercaptoacetamide

1. Quantitate crude nucleopeptide as described (see Basic Protocol 3, steps 8 to 10).
2. Dissolve 50 OD_{260} units lyophilized crude nucleopeptide in 1 mL water.
3. Place at 37°C and add a 20- to 100-fold excess (2 to 10 µL) *N*-methylmercaptoacetamide. Stir occasionally.

4. After 12 hr, monitor the reaction by reversed-phase C18 HPLC (see Basic Protocol 3, steps 1 to 4).

   In general, the methionine-reduced nucleopeptide is eluted after the sulfoxide-containing nucleopeptide (~1 to 2 min).

5. If necessary, add more *N*-methylmercaptoacetamide to achieve complete reduction within 24 to 36 hr.
6. When complete reduction is achieved, lyophilize and proceed to isolate the desired reduced nucleopeptide (see Basic Protocol 3, step 6 or 12).

SUPPORT PROTOCOL 7

DEPROTECTION AND PURIFICATION OF CYSTEINE-CONTAINING NUCLEOPEPTIDES

Additional Materials *(also see Basic Protocol 3)*

Nucleopeptide-support (see Basic Protocol 2, step 7)
1 M 1,4-dithiotreitol (DTT) in peroxide-free dioxane (see recipe)
32% ammonium hydroxide
0.05 M ammonium acetate buffer, pH 7.0

20-mL screw-cap pressure tube, O-ring seal preferred
Disposable polypropylene syringe with polyethylene filter (or cotton)
50-mL round-bottom flask
Peristaltic pump
Sephadex G-10 column

Deprotect cysteine-containing nucleopeptide

1. Open the synthesis column and transfer the nucleopeptide-support to a 20-mL screw-cap pressure tube, preferably with an O-ring seal.
2. Add 5 mL of 1 M DTT in peroxide-free dioxane and 5 mL of 32% ammonium hydroxide, screw the cap on tightly, and incubate 6 to 8 hr at room temperature.

   It is necessary to have a large excess of DTT during the ammonia deprotection step to remove the tert-butylthio protecting group from cysteine and to avoid secondary reactions such as β-elimination of HS-S-tBu. Dimethylaminomethylene-protected guanosine and acetyl-protected cytosine phosphoramidites must be used in combination with tert-butylthio-protected cysteine residues, and cleavage and deprotection must be performed at room temperature for 6 to 8 hr. Refer to safety recommendations and other notes above (see Basic Protocol 2).

3. Filter the solution through a disposable polypropylene disposable syringe with a polyethylene filter or cotton and collect the filtrate in a 50-mL round-bottom flask.
4. Wash the pressure tube three times with MeOH and pass the washes through the syringe into the same flask.
5. Concentrate the combined solution under reduced pressure (in a rotary evaporator with a water aspirator) to a volume of ~2 mL.

Perform gel filtration

6. Set up a purification system as described for MPLC purification (see Basic Protocol 3, step 12), but with a peristaltic pump.
7. Equilibrate a Sephadex G-10 chromatography column with 200 mL of 0.05 M ammonium acetate buffer, pH 7.0.
8. Dilute the crude nucleopeptide sample with 0.05 M ammonium acetate buffer, pH 7.0, to a volume of 5 mL.

9. Remove the supernatant solution from the column cap position. Introduce the nucleopeptide sample solution in the column and close.
10. Set the UV detector wavelength at 260 nm with the appropriate lamp and filter. Turn on the detector and chart recorder. Turn on the peristaltic pump and elute slowly (~1 mL/min) with 0.05 M ammonium acetate buffer, pH 7.0.

    Under the conditions described, the nucleopeptide-containing peak is eluted before the DTT-containing peak. This is because separation is based on molecular weight differences, with the larger compounds being eluted faster.
11. Observing the chromatogram, select the tubes where the nucleopeptide-containing peak is eluted and combine eluents. Lyophilize, analyze by HPLC, and purify as described (see Basic Protocol 3).

    It is important to point out that cysteine-containing nucleopeptides may dimerize very quickly because of oxidation by atmospheric oxygen, yielding a dimer in which two nucleopeptides are bonded through a disulfide bridge between the peptide chains. When two or more cysteine residues are present in the nucleopeptide, polymers can be formed. To prevent oxidation, avoid having samples in solution at room temperature. Keep frozen.

ANALYSIS AND PURIFICATION OF NUCLEOPEPTIDES BY POLYACRYLAMIDE GEL ELECTROPHORESIS

BASIC PROTOCOL 4

For more details on oligonucleotide polyacrylamide gel electrophoresis, see *UNIT 10.4* and *APPENDIX 3B*.

Materials

Urea, ultrapure
38% (w/v) acrylamide/2% (w/v) bisacrylamide (see recipe)
10× TBE buffer (1.3 M Tris, 0.45 M boric acid, 25 mM EDTA)
10% (w/v) ammonium persulfate (store up to 1 week at 4°C)
N,N,N′,N′-Tetramethylethylenediamine (TEMED)
Bromphenol blue
Xylene cyanol
Glycerol
Deprotected crude or purified nucleopeptide (see Basic Protocol 2 or 3)
50% (v/v) aqueous formamide
Stains-all (Aldrich)
Formamide
Concentrated HCl
Isopropanol
3 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
2 M ammonium acetate buffer, pH 7.0
Acetonitrile, HPLC grade
50% (v/v) aqueous methanol (MeOH)

250-mL Erlenmeyer flask
20- and 60-mL syringes
95° and 37°C water baths or heating blocks
Sep-Pak cartridges (Waters) with clamps
Polyethylene disc
Disposable tubes
Lyophilizer

Additional reagents and equipment for polyacrylamide electrophoresis (*UNIT 10.4* and *APPENDIX 3B*)

Set up gel

1. Assemble gel plates, spacers, and combs as described in *APPENDIX 3B* or following manufacturer's instructions.
2. In a 250-mL Erlenmeyer flask, combine the following:

   12.6 g urea
   15 mL 38% (w/v) acrylamide/2% (w/v) bisacrylamide (20% acrylamide)
   1.5 mL 10× TBE buffer
   30 mL water.

   CAUTION: *Acrylamide and bisacrylamide are hazardous. Solutions of acrylamide deteriorate quickly, especially when exposed to light or left at room temperature (see Reagents and Solutions).*

   For oligonucleotides with <25 bases, 20% is the optimum acrylamide concentration (UNIT 10.4).

   To speed dissolution of urea, the gel mixture can be heated before adding TEMED and ammonium persulfate; however, to prevent degradation of acrylamide, do not heat above 55°C. Allow to cool to room temperature before adding TEMED and ammonium persulfate to prevent polymerization while pouring the gel.

3. Add 300 µL of 10% (w/v) ammonium persulfate and 30 µL TEMED. Mix thoroughly and wait 30 sec.
4. Gently pull acrylamide solution into a 60-mL syringe, avoiding bubbles, and pour the gel, allowing it to flow slowly down between the gel plates (see *APPENDIX 3B*).
5. Insert the comb. Dislodge any trapped air bubbles, especially in the wells, by tapping gently on the glass plates.

Set up electrophoresis apparatus

6. After the gel has polymerized (usually 30 to 60 min), remove bottom spacer of gel sandwich and remove extraneous polyacrylamide from around the combs.
7. Fill bottom reservoir of gel apparatus with 0.5× TBE buffer. Place the gel in the electrophoresis apparatus and clamp plates to support.
8. Pour 0.5× TBE buffer into the upper reservoir to ~3 cm above the top of the gel. Using a Pasteur pipet, rinse wells thoroughly with 0.5× TBE buffer to remove stray fragments of polyacrylamide.
9. Preheat gel ~1 hr by running at a constant 750 V.

   The ideal wattage of the gel should generate enough heat so that the gel plates are warm, but not too hot to touch.

Load and run the gel

10. To remove urea that has leached into them, flush the sample wells with 0.5× TBE buffer just prior to loading the gel.
11. Prepare a 5× stock solution with marker dyes:

    0.25% (w/v) bromphenol blue
    0.25% (w/v) xylene cyanol
    30% (w/v) glycerol.

    Dilute to 1× with water before loading onto the gel.

    In a 20% acrylamide gel, migration of bromphenol blue and xylene cyanol will be similar to 6-nt and 22-nt linear single-stranded oligonucleotides, respectively.

12. Dissolve each sample of crude or purified nucleopeptide (0.1 to 0.2 OD_{260} units) in 20 µL of 50% aqueous formamide by vortexing. Microcentrifuge the dissolved samples briefly to collect them at the bottom of the tube.

13. Heat samples at 95°C in covered microcentrifuge tubes. Place on ice.

14. Load 10 µL sample per well under the surface of the buffer and just above the surface of the well.

15. Load 5 µL of 1× marker dye solution (step 11).

16. Run gel immediately at 750 V constant voltage. Maintain a constant gel temperature of ~65°C. Observe migration of marker dyes to determine electrophoresis time.

    Temperatures >65°C can result in cracked plates or smeared bands; temperatures that are too low can lead to incomplete denaturation.

Process gel

17. Prepare a 10% stock dye solution by dissolving 100 mg Stains-all in 100 mL formamide. Adjust pH to 7.3 to 7.4 by adding concentrated HCl. Store at 4°C, protected from light.

18. Mix the following:

    10 mL 10% Stains-all solution
    10 mL formamide
    50 mL isopropanol
    1 mL 3 M Tris·Cl, pH 8.8
    129 mL water.

19. Remove the gel sandwich from the apparatus and carefully place the gel in a shallow pan containing enough staining solution to cover the gel.

20. Let stand 6 hr to overnight, then decant the stain and gently rinse the gel in water.

    If the background is too high, soaking the gel in water and exposing to infrared light can effect some destaining.

21. Photograph the gel under ambient light against a white background. Alternatively, dry using a gel dryer (*APPENDIX 3B*) and scan or photograph.

Purify nucleopeptide by preparative gel electrophoresis

22. Prepare a denaturing polyacrylamide gel as described above and in *APPENDIX 3B*. Prepare twice the amount of reagents as required for analytical gel electrophoresis.

23. Load 2 to 8 OD_{260} units of nucleopeptide in 40 µL of 50% aqueous formamide and perform electrophoresis.

24. Remove the gel and visualize the nucleopeptide bands using a fluorescent TLC plate and UV lamp (*APPENDIX 3B*).

25. Slice the gel on the perimeter of the band with a clean razor blade, and transfer the fragment to a disposable tube. Crush the fragment with a spatula.

26. Add 3 to 4 mL of 2 M ammonium acetate buffer, pH 7.0, and vortex briefly. Let stand with vigorous stirring for 12 to 24 hr in a 37°C water bath. Alternatively, freeze and thaw at least three times to enhance extraction of the product from gel.

27. Mount a Sep-Pak cartridge with a clamp and insert an empty 20-mL syringe on top of the cartridge.

28. Fill the syringe barrel with 10 mL acetonitrile. Insert and depress the plunger to pass the solution through the Sep-Pak cartridge over an elapsed time of 15 to 30 sec.

29. Repeat step 28 with 10 mL water and 10 mL of 2 M ammonium acetate buffer, pH 7.0.

30. Remove the syringe and insert a polypropylene syringe fitted with a polyethylene disc onto the top of Sep-Pak cartridge. Add the 3 to 4 mL buffer and gel (step 26) and let fluid pass through the column by gravity.

31. Wash the disposable tube (from the gel suspension) with 3 to 4 mL water and add to the syringe. Repeat wash three more times.

32. Insert a new disposable syringe and elute nucleopeptide with 5 to 7 mL of 50% MeOH. Collect 1-mL fractions in disposable tubes.

33. Use UV absorbance to identify the fractions where the nucleopeptide has eluted. Lyophilize and quantify.

REAGENTS AND SOLUTIONS

*Use deionized, Milli-Q-purified water (Millipore, 18 m$\Omega \times cm^{-1}$) in all recipes and protocol steps, unless otherwise noted. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acetonitrile, anhydrous

Reflux and distill over calcium hydride powder under an inert (i.e., N_2 or Ar) atmosphere. Store over calcium hydride lumps under an Ar atmosphere. Prepare fresh before each use.

Alternatively, DNA-synthesis-grade low-water-content acetonitrile can be purchased and used as such, or refluxed and distilled from calcium hydride under an inert atmosphere.

Acrylamide/bisacrylamide solution, 38%/2% (w/v)

Dissolve 380 g acrylamide and 20 g bisacrylamide in water to a volume of 1 liter. Filter through a 0.5-µM membrane. Store 2 to 4 weeks at 4°C, protected from light.

Activator solution

Prepare 0.8 M sublimed 1*H*-tetrazole (see recipe) in anhydrous THF (see recipe) using a septum-sealed distillation collection bulb. For a complete dissolution of tetrazole, use an ultrasonic bath. Prepare fresh before each use.

Dichloromethane (DCM), anhydrous

Reflux and distill dichloromethane over reagent-grade phosphorus pentoxide under an inert (N_2 or Ar) atmosphere. Store over calcium hydride lumps under Ar atmosphere. Prepare fresh before each use.

DCM, neutralized

Pass DCM through a basic alumina column in order to eliminate acid traces. Store up to one week at 20°C.

N,N-Diisopropylethylamine (DIPEA), anhydrous

Place under calcium hydride lumps under an Ar atmosphere for at least one night before use. Prepare fresh before each use.

Dioxane, peroxide free

Pass dioxane through a basic alumina column in order to eliminate all traces of peroxide. Store until a positive peroxide test is obtained (mix equal volumes of 5% (w/v) KI aqueous solution and dioxane).

A yellowish color indicates the presence of peroxides. No yellowish color should be observed if the solvent is peroxide-free.

Ninhydrin reagent A

Dissolve 40 g phenol in 10 mL absolute EtOH. Mix 2 mL of 2 mM potassium cyanide and 100 mL pyridine freshly distilled over ninhydrin. Mix each solution separately with 4 g Amberlite MB-3 resin (Merck) for 45 min. Filter and mix both solutions together to obtain the desired reagent A. Store up to 1 to 2 years at 4°C.

Ninhydrin reagent B

Dissolve 2.5 g ninhydrin in 50 mL absolute EtOH. Keep the solution protected from light in an amber glass container (up to 1 to 2 years at 4°C).

Oxidizer solution

Dilute 5.0 to 6.0 M anhydrous *tert*-butylhydroperoxide in decane (Aldrich or Fluka) to a final concentration of 1 M in anhydrous DCM (see recipe). Prepare fresh before each use.

Pyridine, anhydrous

Reflux and distill pyridine over reagent-ninhydrin powder under an inert (i.e., N_2 or Ar) atmosphere. Reflux and distill again over calcium hydride powder. Place over calcium hydride lumps under an Ar atmosphere. Prepare fresh before each use.

Tetrahydrofuran (THF), anhydrous

Dry peroxide-free tetrahydrofuran (see recipe) by continuous reflux and distillation over sodium metal and benzophenone under inert atmosphere until purple color persists. Prepare fresh before each use.

THF, peroxide free

Pass THF through a basic alumina column in order to eliminate peroxides. Store until a positive test is obtained (see peroxide-free dioxane). Protect from light.

1H-Tetrazole, sublimed

Transfer a commercial solution of 1*H*-tetrazole in acetonitrile into a round-bottom flask (this can be mixed with unused wet solutions of tetrazole from the DNA synthesizer). Evaporate to dryness under reduced pressure using a rotary evaporator with a water aspirator. Use a sublimation apparatus to sublime the remaining solid to obtain pure crystalline 1*H*-tetrazole. Store under Ar up to 5 years at ambient temperature.

CAUTION: *1H-Tetrazole is an explosive compound. Never heat near the melting point (157°-159°C). Wear safety glasses and gloves during sublimation procedure. Manipulate with caution in a well-ventilated fume hood.*

1H-Tetrazole is no longer commercially available in solid form. Solutions of 1H-tetrazole in acetonitrile may be purchased from typical organic chemical suppliers such as Aldrich.

COMMENTARY

Background Information

The methodologies for the preparation of various kinds of peptide-oligonucleotide conjugates have been reviewed by different authors (Tung and Stein, 2000; Zubin et al., 2002; UNIT 4.5). The research published since the last review report on adaptations, modifications, problems, or improvements of previously described methods (e.g., Chen et al., 2002, 2003; Drioli et al., 2002; Kachalova et al., 2002; Viladkar, 2002; Zatsepin et al., 2002), but no new synthesis design has been described. For these reasons, as well as for space limitations, only a few comments on the preparation of nucleopeptides and on the use of stepwise solid-phase methodology to obtain peptide-oligonucleotide conjugates will be made here (i.e., only representative examples will be cited).

All the methodologies used to prepare hybrids in which the two components are directly linked through phosphate diesters (here referred to as nucleopeptides) share a common outline: the key linking phosphodiester bond is

created having all the nonparticipating functional groups protected.

This key phosphate has been formed using different chemistries that yield phosphodiester bonds, namely the phosphate triester (Kuyl-Yeheskiely et al., 1989; Ueno et al., 1993), the *H*-phosphonate (Kuyl-Yeheskiely et al., 1987), and the phosphite triester (Dreef-Tromp et al., 1992a; Sakakura and Hayakawa, 2000; Debéthune et al., 2002a; Jeyaraj et al., 2002) approaches. Synthesis of nucleopeptides has been carried out in solution and on solid supports, both by convergent (Dreef-Tromp et al., 1992a; Robles et al., 1995; Sakakura and Hayakawa, 2000) and stepwise strategies (Debéthune et al., 2002a). Protection schemes have included a large variety of permanent protecting groups that are either chemically or enzymatically (Flohr et al., 1999) removable.

Many nucleopeptides have been prepared since the pioneering work of Z.A. Shabarova in the sixties (Shabarova, 1970). In 1992, van Boom's group was able to obtain a nucleopeptide with up to ten nucleosides (Dreef-Tromp et al., 1992a) and two trifunctional amino acids besides the linking residue. The methodology described here has allowed the synthesis of nucleopeptides containing up to five amino acids with functionalized side chains (besides the linking residue), and to obtain a nucleopeptide with thirteen amino acids and fifteen nucleosides.

In the authors' opinion, one of the main advantages of this stepwise solid-phase methodology is that it circumvents the need to manipulate protected peptides. These are often highly insoluble in most organic solvents, which renders both the purification and the phosphitylation (or phosphorylation) difficult.

Moreover, most of the monomers required can be purchased from commercial suppliers. Besides the trifunctional amino acid derivatives whose synthesis is described here, the only nucleoside synthons that should have to be synthesized in the laboratory are the 5′-phosphoramidites of 2′-deoxycytidine and 2′-deoxyguanosine. The commercially available 5′-phosphoramidites of these nucleosides are only suitable for the preparation of nucleopeptides with 5′-oligonucleotide-homoserine or tyrosine linkages, and their standard nucleobase-protecting groups are not compatible with base-labile nucleopeptides.

Finally, it is worth emphasizing that compared to other methods the use of an amino acid to attach the two moieties has the advantage of allowing one to place the linking unit at any position in the peptide chain, with no need to prepare a specially designed support. The only exception to this assertion, as previously mentioned, is that homoserine cannot be placed at the C-terminal position. The acid treatment that removes the Boc group simultaneously eliminates its DMTr hydroxyl-protecting group, and the propensity for homoserine to lactonize would easily provoke cleavage of the peptide-resin bond, resulting in loss of peptide chains.

As previously stated, peptide-oligonucleotide conjugates that are not directly linked or are linked through amide bonds have also been synthesized using stepwise procedures. In most cases, hybrids have been assembled onto insoluble matrices, but the modifiable solubility of polyethylene glycol has also been exploited (Drioli et al., 2002). Peptide assembly is more often carried out first, but synthesis starting with oligonucleotide elongation has also been reported (Bergmann and Bannwarth, 1995; Sarracino et al., 1998). In general, there is no need for concern about the stability of the linkage between the two moieties, but some degradation of the target molecule during the final deprotection treatment has been found to be associated with the use of immobilized trifunctional linkers (Basu and Wicsktrom, 1995).

It is important to note that the protection scheme proposed here for the preparation of nucleopeptides is of general application and can be extended to the synthesis of any peptide-oligonucleotide conjugate. In addition, when the target molecule contains trifunctional amino acids that may suffer side reactions, and/or base-labile linkers are used for their assembly, the recommendation to use the most labile set of permanent protecting groups also applies.

The most common applications of peptide-oligonucleotide conjugates have been discussed earlier (see unit introduction). Such molecules can also find application in structural studies (Ho et al., 1999; Gómez-Pinto et al., 2003), in studies on the structural requirements of an enzyme (Debéthune et al., 2002b), or in studies on the behavior of metal complexes (Civitello et al., 2001; Marchán et al., 2001).

Hopefully, access to user-friendly synthesis procedures and the future development of newer, more convenient procedures will inspire an increasing number of laboratories to prepare more conjugates, and thus enlarge their fields of application.

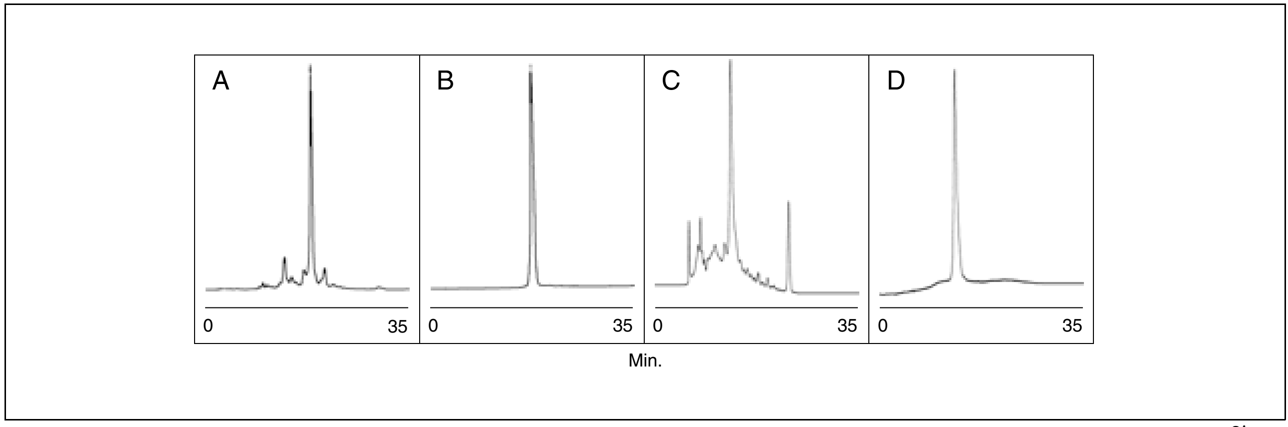

Figure 4.22.9 Examples of HPLC profiles of nucleopeptides: (**A**) crude and (**B**) purified Ac-Lys-Trp-Lys-Hse($p^{3'}$dG-CATCG)-Ala-OH, and (**C**) crude and (**D**) purified Ac-Tyr($p^{3'}$dTTTCAGAAAATCTAG)-Leu-Asp-Pro-Arg-Ile-Thr-Val-OH. See Basic Protocol 3 for gradient conditions, eluents, and column employed.

Critical Parameters

As previously pointed out, the most critical point in nucleopeptide synthesis is the choice of protecting groups, which must be accompanied by appropriate selection of the final deprotection conditions (see Strategic Planning and Table 4.22.1).

It is also important to ensure that oligonucleotide elongation is carried out on a homogeneous immobilized peptide. The amino acid composition should be checked before the oligonucleotide synthesis is begun. Depending on the peptide length and composition, HPLC and MS analysis of the crude product obtained after deprotection of an aliquot of peptide-resin may also be worthwhile.

If large nucleopeptides or conjugates are to be prepared, leaving the DMTr group after incorporation of the last nucleoside synthon may facilitate the HPLC analysis and purification of the crude product. Two purification runs, both by HPLC or by combining HPLC and PAGE, may sometimes be required.

Troubleshooting

Large nucleopeptides, especially depending on the amino acid composition, may show complex HPLC profiles. HPLC analysis at higher temperature (50° to 55°C) may reduce conformational equilibria, and in some cases simplify the HPLC profile of the crude product. As previously suggested, analysis by PAGE under denaturing conditions may also be a good alternative.

Anticipated Results

If peptide elongation has proceeded satisfactorily, the expected peptide should be the major peak in the crude nucleopeptide after cleavage and deprotection.

The coupling yield of the first nucleoside phosphoramidite is variable, with an average value of 70%. Nearly quantitative yields, as in any other oligonucleotide synthesis, are obtained in the subsequent couplings.

The cleavage yield with concentrated ammonia/dioxane treatment at 55°C usually ranges from 60% to 80%. An additional treatment with TBAF, if allowed by the nucleopeptide structure, leaves <5% of the nucleopeptide on the resin.

The purity of the crude nucleopeptide varies depending on the nucleopeptide size and composition. In the HPLC profiles shown in Figure 4.22.9, the main peak (expected product) accounts for ~75% of the total area in the case of the tryptophan-containing nucleopeptide, and ~60% in the arginine-containing one. MALDI-TOF-MS analysis of the crude sample allows confirmation that it contains the target product.

Purification can usually be accomplished in a single run by either reversed-phase MPLC or HPLC on semipreparative C18 columns.

Overall yields (nucleopeptide assembly, deprotection, and purification) will also vary depending on the homogeneity of the crude product and whether purification requires one or two steps. The arginine-containing nucleopeptide, for instance, is an example of a nucleopeptide of medium difficulty, and was obtained in 17% yield.

Time Considerations

The preparation of nucleopeptides using these procedures may be a time-demanding task if trifunctional amino acid derivatives, as well as

the handle linking the hybrid molecule to the resin, have to be prepared. With all of the precursors, reagents, and solutions in hand, the synthesis and purification of a nucleopeptide containing ten amino acids and a 10-mer oligonucleotide can be accomplished in ~3 weeks, depending, for instance, on the availability of techniques such as amino acid analysis to assess that correct yields have been obtained, or on whether peptide purity is checked before proceeding with oligonucleotide elongation. The time required to purify the target molecule may vary depending on the scale of synthesis and, obviously, the quality of the crude product. A tentative schedule would be the following:

Week 1: Preparation of solid support, manual synthesis of peptide chain, and analysis of peptide-resin.

Week 2: Oligonucleotide elongation, deprotection and cleavage, and analysis of the crude nucleopeptide to set up the purification conditions.

Week 3: Purification of the nucleopeptide and full characterization of the product (amino acid analysis after acid hydrolysis or enzymatic digestion, determination of the relative proportion of nucleosides after enzymatic digestion, and mass spectrometric analysis).

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Review covering synthesis and applications of peptide-oligonucleotide conjugates.

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The authors wish to acknowledge the contribution of Dr. Jordi Robles, who set up the basic nucleopeptide stepwise solid-phase synthesis methodology, in addition to the contributions of many other people over the years.