Attachment of Nucleosides and Other Linkers to Solid-Phase Supports for Oligonucleotide Synthesis

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ABSTRACT

Specific step-by-step instructions for conversion of 5′-*O*-(4,4′-dimethoxytrityl)- and base-protected nucleosides and other mono-*O*-(4,4′-dimethoxytrityl)-protected diols to their hemisuccinate esters and their coupling to CPG (controlled-pore glass) supports bearing aminopropyl or long chain aminoalkyl groups are presented. Additional guidelines are provided for selecting a coupling protocol and performing in-process control. *Curr. Protoc. Nucleic Acid Chem.* 52:3.2.1-3.2.23. © 2013 by John Wiley & Sons, Inc.

Keywords: nucleic acid chemistry • oligonucleotides • nucleosides and nucleotides • solid supports

INTRODUCTION

The first step in any solid-phase synthesis is the covalent attachment of the first building block (i.e., a nucleoside or a non-nucleosidic linker) to the surface of an insoluble support. For oligonucleotide synthesis, this process is performed separately from the automated coupling steps used for chain elongation because of the different coupling chemistry involved. Although prederivatized solid supports are commercially available from a number of sources, preparation of these materials in the lab is an affordable alternative to their purchase. Many special nonnucleosidic supports and those containing minor nucleosides are either not commercially available or very expensive. The objective of this unit is to help a chemist with no or only limited prior experience in the field to properly approach the preparation of derivatized solid supports for oligonucleotide synthesis. The simplicity of the procedures and ready availability of starting materials allow one to produce amounts of solid supports sufficient for a great number of oligonucleotide syntheses at significant savings.

This unit provides protocols for all steps immediately involved in the preparation of conventional solid supports for routine oligonucleotide synthesis. First, aminopropyl controlled pore glass **3** (aminopropyl CPG) is prepared starting from Native CPG **2** with underivatized surface (Basic Protocol 1 and Fig. 3.2.1). The second required component is prepared next by acylation of a mono-*O*-(4,4′-dimethoxytrityl)-protected diol **4** (i.e., 5′-*O*-DMT- and base-protected nucleoside) with succinic anhydride **5a** (Basic Protocol 2 and Fig. 3.2.2). Examples of hemisuccinates **7**-**9** most frequently used in the current oligonucleotide production are shown in Figure 3.2.2. Finally, the hemisuccinate building blocks **6a** thus obtained are attached to aminopropyl CPG **3** support (Basic Protocol 3 and Fig. 3.2.3). Almost any moiety having one hydroxy group protected with a DMT group and another, unprotected hydroxy group, can be attached in the same manner. Should one require a more hydrolytically labile ester function for a more rapid release of oligonucleotides from solid supports, a diglycolyl moiety can be introduced in place of the succinate linker by the action of diglycolic anhydride **5b**. Examples of diglycolate

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hemiesters **10** (Pon and Yu, 1997a) and **11** (Guzaev and Manoharan, 2001) are shown in Figure 3.2.2. The protocol is also not limited to aminopropyl CPG and can be used for the attachment to long-chain alkylamine (LCAA) CPG and macroporous aminomethyl polystyrene with no modifications. The Alternate Protocol describes an expedited version of Basic Protocol 3. Protocols for quantitative determination of the amino and the 4,4′-dimethoxytrityl (DMT) groups attached to a support (Support Protocols 1 to 3) and for qualitative monitoring of the presence of the amino groups on the surface (Support Protocol 4) are also included.

The procedures described use only very basic equipment so that they can be conducted virtually in any industrial or academic chemical laboratory without any additional investment in labware. Basic training in synthetic organic chemistry should be sufficient for the proper execution of all protocols.

STRATEGIC PLANNING: SELECTING A LINKER ARM AND COUPLING PROTOCOL

It is important to bear in mind that no single solid support satisfies the requirements of all synthetic applications. It is the intended use of the solid support that determines its properties and features. Prior to embarking on the preparative work, a few basic parameters of the intended application should be considered: the scale of oligonucleotide synthesis, the length of oligonucleotides to be assembled, the linker to be used, and the compatibility of the solid support material and the linker strategy with any nonstandard structural features in the oligonucleotides to be synthesized.

The Scale of Oligonucleotide Synthesis Determines the Solid Support Material

Rigid, macroporous CPG particles described in this unit are readily available in a variety of pore sizes. When selecting the appropriate kind of CPG for the synthesis, it is worth bearing in mind that the pore size and the specific surface area are interdependent variables. The smaller the pore size is, the greater the surface area is and the higher the linker loading can be obtained. Typically, the maximum loading of aminopropyl CPG with moieties comparable to nucleosides in size does not exceed 1.5 μmol/m^2. Thus, the solid supports prepared from CPG 500, 1000, 1500, and 2000 Å can be loaded to 80 to 90, 50 to 60, 35 to 45, and 20 to 30 μmol/g, respectively, although aiming at the highest permissible loading is not necessarily the best choice. The CPG supports perform best when loaded to less than 90% of their maximum capacity. CPG supports of all pore sizes are quite suitable for a broad range of small-scale syntheses of oligonucleotides (less than 25 μmol). Selected brands of high cross-linked, macroporous polystyrene (MPPS) with a typical loading of 10 to 45 μmol/g perform well in small scale applications, too. On scales of 25 to 100 μmol, high cross-linked, macroporous polystyrene (MPPS) loadable to 200 to 400 μmol/g is a good choice, while the use of CPG 500 and, to a limited extent, of CPG1000 still remains an option. Large scale synthesis (over 100 μmol) is best performed on MPPS.

The Length of Oligonucleotides Determines the Pore Size

The intended length of oligonucleotides will determine the pore size of the solid support matrix. To synthesize oligonucleotides shorter than 50 bases, CPG500 Å is a good choice, while CPG supports with pore sizes of 1000, 1500, 2000, and 3000Å allow the preparation of about 80, 100, 150, and 200-mer oligonucleotides. While enjoying a high loading capacity, most MPPS are capable of supporting the synthesis of oligonucleotides shorter than 40- to 50-mers.

The Types of Linker Chemistry

A comprehensive review on the use of linkers in oligonucleotide chemistry is provided in *UNIT 3.01*. Broadly, there are three types of linkers currently used in oligonucleotide synthesis.

Universal linkers for high-throughput and large-scale synthesis

Universal linkers are nonnucleosidic linkers that serve as anchoring sites for the attachment of the first nucleoside phosphoramidite. Upon the completion of the synthesis, the phosphodiester bond joining the universal linker to the 3′-terminal nucleoside is hydrolyzed at the 3′-*O*-P bond (Guzaev and Manoharan, 2003). This releases the standard, 3′-dephosphorylated oligonucleotide with a free 3′-hydroxy group. As the name suggests, universal linkers can be used for the synthesis of oligonucleotides having any nucleoside at the 3′-terminus. Use of universal solid supports bearing the linker **9** and other high-performance universal linkers is highly recommended for facilities wishing to increase their daily throughput, particularly those operating in the plate format of oligonucleotide synthesis and on large synthetic scale. Although the universal building blocks are poorly available commercially, the sufficient information for their synthesis is available in the public domain. However, the loaded universal solid supports are commercially available from several sources.

Nucleosidic linkers for incorporation of rare nucleosides at the 3′-end

Nucleosidic linkers **7** and the respective solid supports dominated oligonucleotide synthesis until about 10 years ago. They are still used, although to a lesser extent. One case where nucleosidic solid supports still prevail over the universal supports is the synthesis of base-labile oligonucleotides (phosphotriester and methylphosphonate analogs or those incorporating base-labile nucleoside residues). Linker arms more labile than succinyl (diglycolyl linker **10** and Q-linker) are particularly useful for these applications (Pon and Yu, 1997a). Also, oligonucleotides where the 3′-terminal nucleoside is a rare, expensive, or novel nucleoside analog are best synthesized on the respective nucleosidic solid support. Indeed, it is more economical to attach a nucleoside to the solid phase than to use it as a phosphoramidite in a large excess.

Specialty linkers for 3′-derivatized oligonucleotides

Specialty linkers form a large and diverse group of linkers that do not belong to universal or nucleosidic linkers. The solid supports with specialty linkers attached are used to synthesize oligonucleotides with a modified 3′-terminus: 3′-phosphate (linker **11**), amino, or mercapto functional group, a fluorescent label or quencher, cholesterol, or any other hydrophobic residue to name a few.

The Nonstandard Features: Impact on the Properties of Oligonucleotides

When the incorporation of nonstandard building blocks in oligonucleotides is planned, the compatibility of the solid support to be used with the final deprotection strategy and the properties of the product deserve special consideration. For instance, a nucleoside analog or a dye might be of limited stability under the standard basic conditions of deprotection. Its use will require a linker cleavable under mild basic conditions or even under UV-irradiation. A building block might occupy too large a space to be accommodated even by wide-porous CPG without using an additional spacer arm. In this case, the use of LCAA CPG is highly recommended. A highly hydrophobic building block might prevent the elution of an oligonucleotide conjugate from polystyrene solid supports, but the synthesis may be quite successful on CPG supports. This by no means inclusive list of examples demonstrates the possible complications in the preparation of nonstandard oligonucleotides. While no single protocol can account for all scenarios, prediction and overcoming of synthetic complications remains the challenge to be met by a skillful researcher.

PREPARATION OF AMINOPROPYL CPG

This protocol describes the attachment of aminopropyl arm to the native CPG **1** by action of 3-(triethoxysilyl)-1-propanamine **2** (Fig. 3.2.1). Without specialized (and expensive) equipment, the procedure is scalable up to 3.75 kg of CPG to be conducted in a 22-L round-bottom flask or down to about 5 g. On scales smaller than 5 g of CPG, one can carry out amination in screw-capped test tubes with occasional mixing by hand.

Materials

Water
Native CPG beads (aminopropyl- or LCAA CPG or aminomethyl macroporous polystyrene may be directly subjected to the loading; see recipe)
3-(Triethoxysilyl)-1-propanamine, reagent-grade (**2**)
Methanol, reagent-grade *or* ethanol
Ethyl acetate, optional

1-L Round-bottom flask with overhead stirrer, reflux condenser, and thermometer
Heating mantle or oil bath, temperature regulator
Buchner funnel
Bunsen flask
Sintered glass *or* Whatman no. 1 filter paper
Vacuum oven *or* rotary evaporator

Additional reagents and equipments for picrate test (see Support Protocol 1)

1. Place 350 mL water in 1-L round-bottom flask equipped with reflux condenser, mechanical stirrer, thermometer, and heating mantle or oil bath. Switch stirring on and slowly add 100 g native CPG via a funnel for solid materials.

   The rate of the addition should be sufficiently slow to avoid the formation of large chunks of CPG.

   When 3 L or larger flasks are used, wide sidenecks of the largest commercially available size are recommended.

   As CPG is being added, the suspension becomes thicker. Gradually increase the rate of stirring as needed to keep CPG suspended. Do not allow CPG to settle, as it might be difficult to re-start the stirring, particularly on larger scales.

2. Heat the suspension to 70° to 75°C and add 46.5 g 3-(triethoxysilyl)propylamine (**2**) in one portion by a slow stream.

   The addition of the silylating agent initiates an exothermic reaction. The temperature, however, will not rise to the boiling point of the liquid phase.

3. Heat the suspension to 85° to 90°C and maintain the stirring at this temperature for at least 8 hr.

   The mixture may be left heated and stirred overnight without compromising the quality of the product.

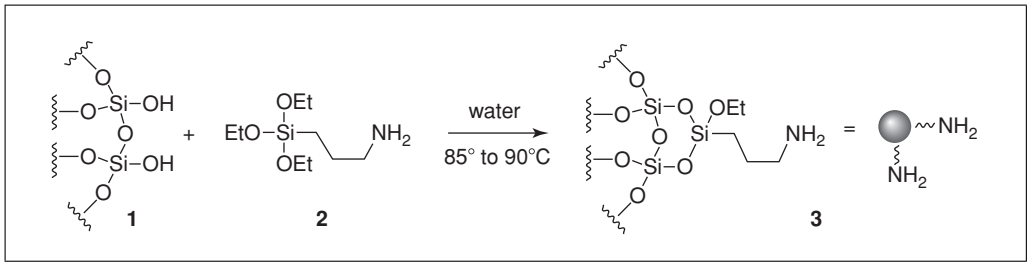

Figure 3.2.1 Preparation of Aminopropyl CPG, **3**, by aminoalkylation of Native CPG, **1**, with 3-(Triethoxysilyl)-1-propanamine, **2**.

4. Allow the mixture to cool and filter the solid off using a Bunsen flask equipped with sintered glass filter or Buchner funnel with filter paper. Wash CPG on the filter repeatedly with distilled water until the washings show pH 7 or slightly lower.

   Do not attempt to substitute washing with water by neutralization with any acid or buffer.

5. Wash the aminated CPG two to three times, each time with 300 mL methanol or ethanol (denaturated ethanol is acceptable).

   To facilitate drying, an optional wash with ethyl acetate (2 × 300 mL) may be used.

6. Dry aminopropyl CPG in a vacuum oven at 70° to 80°C or on rotary evaporator at 50° to 60°C using an adapter equipped with a sintered glass frit.

   Prior to placing a container with aminopropyl CPG in the oven, cover the neck with filter paper or a paper towel secured by a rubber band.

7. Optionally, measure the loading of aminopropyl CPG with amino groups using picrate method (see Support Protocol 1 below).

PREPARATION OF DMT-PROTECTED HEMIESTER BUILDING BLOCKS

BASIC PROTOCOL 2

This protocol (Fig. 3.2.2) describes the preparation of DMT-protected hemiester building blocks **6a** (*N*-protected 5′-*O*-DMT-nucleoside-3′-*O*-hemisuccinate, **7** or hemisuccinates of other mono-DMT-protected diols, **8** and **9** and many others). The reaction time depends on the character of the hydroxy group to be succinylated and whether the catalyst, DMAP or *N*-methylimidazole (NMI), is present in the reaction mixture.

The primary hydroxy groups react in 24 to 48 hr in the absence of any catalysis.

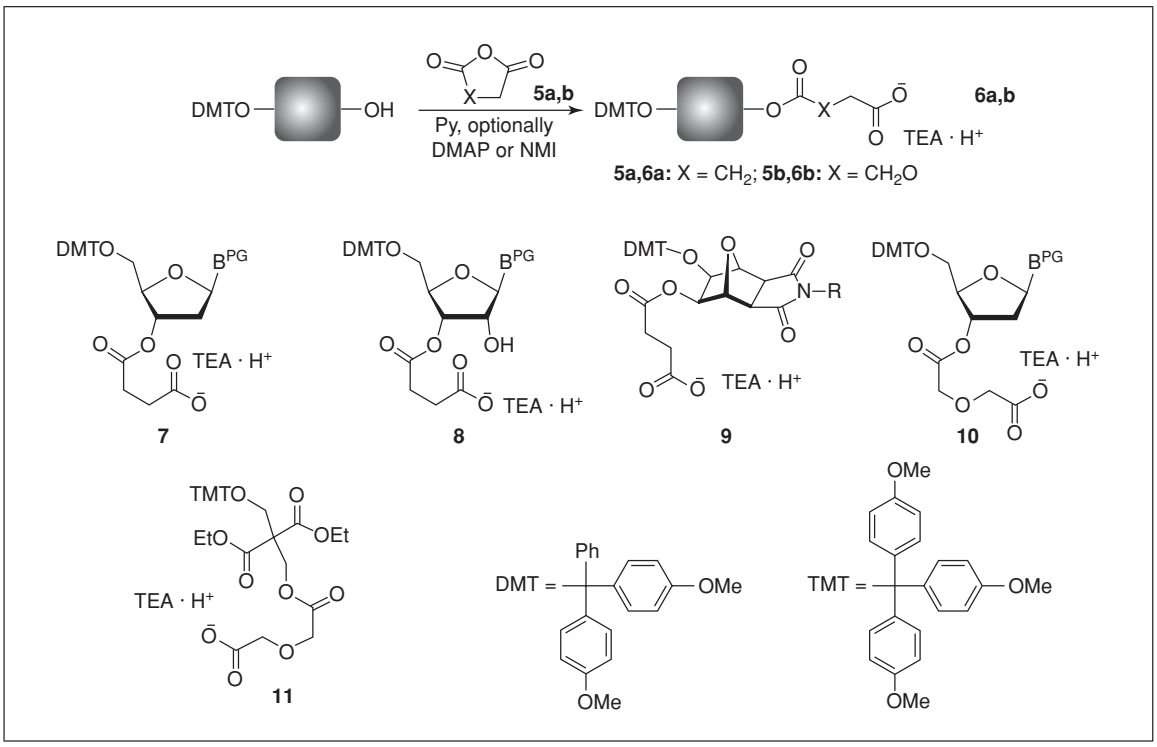

Figure 3.2.2 Conversion of mono-DMT-protected diols, **4**, to the respective hemisuccinates, **6a**, and hemidiglycolates, **6b**. Examples shown below are commonly used hemisuccinates of protected 2′-deoxynucleosides, **7**, protected ribonucleosides, **8**, and universal linker, **9**, and hemiglycolates of protected 2′-deoxynucleosides, **10**, and linker **11** for 3′-phosphorylation of oligonucleotides.

The unhindered secondary hydroxy groups similar to those in 2′-deoxynucleosides require 4 to 6 hr and 10 to 12 hr in the presence of DMAP (0.2 and 0.1 eq. to the building block, respectively). However, the product often requires purification by chromatography on silica gel. The reaction time is longer, 2 and 4 days when NMI (0.1 and 0.2 eq., respectively) is used. In the absence of a catalyst, it may be necessary to allow up to 6 to 7 days for the complete succinylation. However, the products from the uncatalyzed reaction or that catalyzed with NMI normally do not require any chromatographic purification.

The succinylation of a tertiary or otherwise sterically hindered hydroxy group proceeds even more slowly and should be monitored carefully for completion of the reaction.

The uncatalyzed protocol is optimized for a minimum labor although the total turn-around time is rather long. Should the turn-around time be critical, a more expedited and more laborious protocol that uses the catalysis by DMAP and requires purification of the hemisuccinate on a silica gel column is recommended.

Hemiesters of diglycolic acid, **10**, **11**, and similar, can be obtained by following this protocol by the replacement of succinic anhydride **5a** by diglycolic anhydride **5b**.

Materials

N-protected 5′-*O*-DMT nucleoside or mono-DMT-protected diol
Succinic anhydride, reagent grade
4-Dimethylaminopyridine (DMAP), optional
N-methylimidazole (NMI), optional
Anhydrous pyridine (see recipe)
Triethylamine, reagent grade
Methanol, reagent grade
Dichloromethane, reagent grade
0.1 M Triethylammonium acetate prepared by neutralization of 0.1 M aqueous acetic acid with triethylamine to pH 7.5
0.5 M citric acid
Na_2SO_4, anhydrous, granulated

Round-bottom flasks
Magnetic stirrer and stirring bar
Silica gel TLC plates
Heating gun or heating plate
Ice bath
Rotary evaporator
Separatory funnel
Erlenmeyer flask
Oil pump

Additional reagents and equipment for thin-layer chromatography (TLC; *APPENDIX 3D*)

1. Combine the following in a round-bottom flask of an appropriate size:

   N-protected 5′-*O*-DMT nucleoside or mono-DMT-protected diol (10 mmol);
   Succinic anhydride (3.01 g, 30 mmol);
   4-Dimethylaminopyridine (DMAP; 240 mg, 2 mmol or 120 mg, 1 mmol), optional;
   N-methylimidazole (NMI; 164 mg, 2 mmol or 82 mg, 1 mmol), optional;
   Anhydrous pyridine (20 mL);
   Magnetic stir bar.

2. Stir the mixture at room temperature for an appropriate period of time (see recommendations above).

3. Check the progress of the reaction periodically starting from 4 hr (reaction catalyzed with DMAP) or the following morning (NMI-catalyzed and uncatalyzed reactions) by thin-layer chromatography (TLC) on silica gel (*APPENDIX 3D*).

   The analysis requires a TLC plate 6- to 10-cm long. Prepare eluent by mixing triethylamine, methanol, and dichloromethane in a ratio of 5:5:90, respectively.

   To neutralize silica gel, prerun the TLC plate in the eluent with no samples applied and dry the plate with warm air.

   Withdraw an aliquot of the reaction mixture (10 to 20 μL) and dilute 10 times with acetonitrile.

   Apply at least three spots on the base line of the dried plate: the diluted sample of the reaction mixture, the starting nucleoside, and a mixed spot where both the starting material and the reaction mixture are applied.

   Pyridine is a strong eluent and a relatively high-boiling solvent. Its presence in the reaction mixture may severely alter the separation. To reduce the amount of pyridine in the spots already applied on the plate, apply toluene 4 to 5 times to each spot, each time drying the spots with air.

   Elute the plate until the eluent reaches the level of 2 to 5 mm from the top of the plate.

   Dry the plate and heat on a heating plate or with a heat gun until orange-colored spots are clearly visible. The analysis should show no starting material left, with the strong spot of the lower-eluting product. If the reaction is incomplete (any starting material is still present), give it additional time to run to completion.

4. After the reaction is complete, quench the excess of succinic anhydride as follows:

   a. Place the magnetically stirred flask in an ice bath and let the reaction mixture cool.
   b. Add water (2.2 mL, 120 mmol) followed by triethylamine (16.7 mL, 120 mmol) and stir for 3 hr.

5. Work up the reaction mixture to isolate the product as follows:

   a. Add 6 mL triethylamine and evaporate the mixture on a rotary evaporator keeping the temperature in the water bath below 40°C.
   b. Co-evaporate two times with a mixture of 50 mL toluene and 5 mL triethylamine to a thick, foaming syrup.
   c. Dissolve the residue in 200 mL dichloromethane and wash the solution three times, each time with 40 mL of 0.1 M triethylammonium acetate, pH 7.5, in a separatory funnel.
   d. Wash the solution with 50 mL of ice-cold 0.5 M citric acid.
   e. Collect the extract in an Erlenmeyer flask, add 6 mL triethylamine, and dry the extract over Na_2SO_4.
   f. Evaporate the extract to solid foam and dry on an oil pump.
   g. Check the purity by TLC as described above.

      When the purity is insufficient, which often happens in DMAP-catalyzed reactions, purify the product on a silica gel column using, typically, a gradient from dichloromethane to triethylamine-methanol-dichloromethane (5:15:85).

BASIC PROTOCOL 3

ATTACHMENT OF HEMISUCCINATES OF DMT-PROTECTED BUILDING BLOCKS TO AMINOPROPYL CPG

The protocol below describes the regular loading of aminopropyl CPG on a 100-g scale using a slight excess of a hemisuccinate building block **6a** (Fig. 3.2.3). However, the preparation may be scaled up to several kilograms or down to 1 g without any changes in molar ratios of the reagents. On a small scale (100 mg to 1 g), it is advisable to use 1.5 eq. of the activated succinate with respect to the desired loading. The protocol is designed for the labor- and material-efficient operation to obtain solid supports loaded to within ±5% with respect to the specification.

The procedures are also applicable to preparation of ribonucleoside solid supports. When a ribonucleoside is attached to an insoluble support, it is of no consequence whether the nucleoside is connected via the 2′- or 3′-hydroxyl group. Therefore, ribonucleosides with a single unprotected 2′-hydroxy group, **8**, their 3′-unprotected isomers, or mixtures of the 2′- and 3′-isomers can be used equally. In either case, after attachment of the nucleoside succinate to the support, the unprotected hydroxyl group is acetylated by the same capping step used to acetylate unreacted amino groups.

Hemiesters of diglycolic acid, **6b** (**10**, **11**, and similar), can be attached to solid supports by following this protocol with no modifications other than the replacement of the linker.

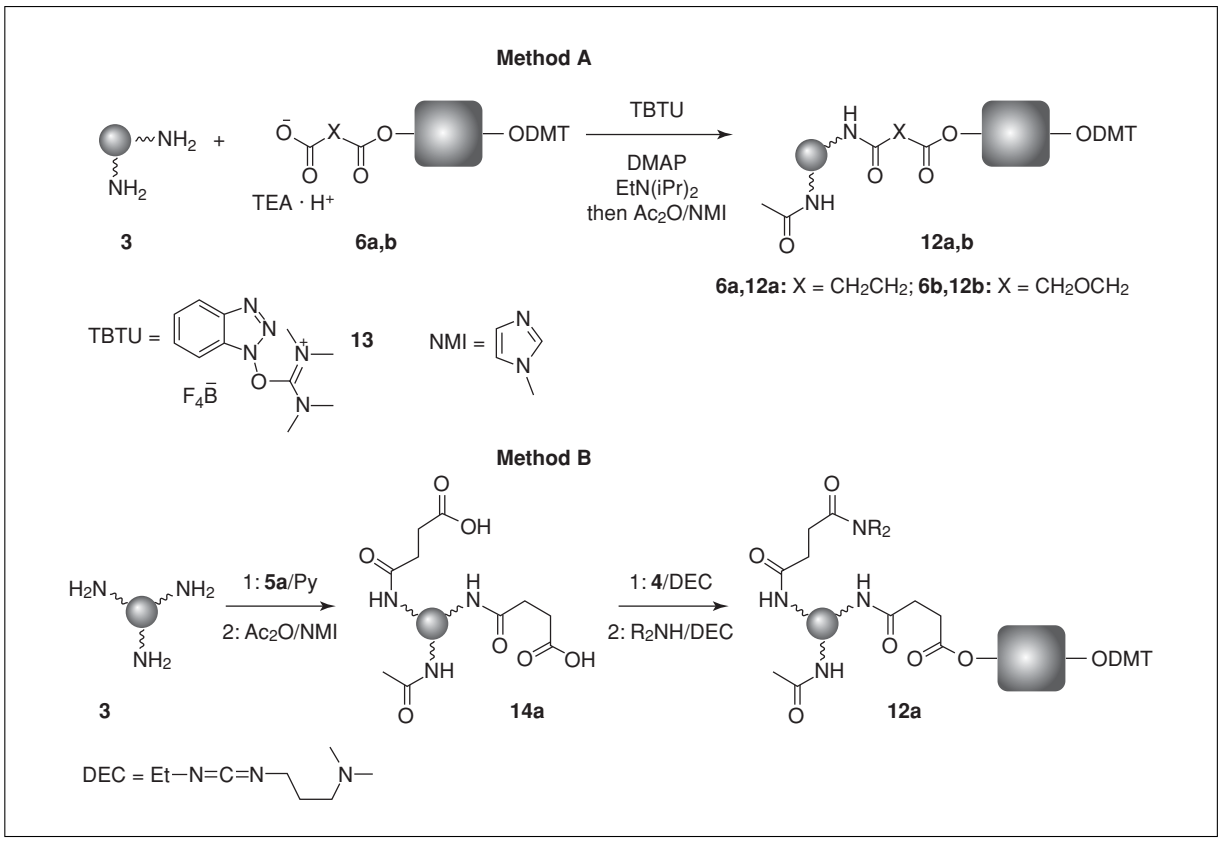

Figure 3.2.3 Preparation of solid supports **12a** and **b** using methods A and B (see Background Information). Method A described in detail in this unit, uses attachment of hemisuccinates **6a** and hemiglycolates **6b** to aminoalkyl-derivatized solid supports **3** with the aid of TBTU as a coupling agent. In the second step, the unreacted amino groups are capped with acetic anhydride/NMI. Protocols for practical implementation of method B can be found in various references (Damha et al., 1990; Pon et al., 1988; also see *UNIT 3.13*).

If the amino group loading of starting support is not known, it is recommended that this important value be determined (see Support Protocol 1) before the attachment of linkers.

Hemisuccinates of linkers may contain amounts of triethylamine different from 1 equivalent. The molar content of triethylamine in most DMT-protected hemisuccinates can be conveniently determined by recording ^{1}H NMR spectrum of the hemisuccinate and integrating peaks of methyl groups in triethylamine (t, δ ~1.1-1.2 ppm) and in DMT (s or d, δ ~3.8-3.9 ppm). The molar ratio x of triethylamine to the DMT-protected species is then calculated as:

$$x = \frac{\text{Integral intensity of Me in TEA}}{1.5 \times (\text{Integral intensity of Me in DMT})}$$

The effective molecular mass of the hemisuccinate is then calculated as M × x$C_6H_{15}N$ where M is molecular mass of hemisuccinate free acid.

Regardless of whether aminopropyl CPG and hemisuccinate were prepared in-house or they were of commercial origin, conduct a test loading using a small amount of material (1.5 to 2 g of aminopropyl CPG) and determine the loading prior to committing to a larger scale preparation.

Materials

Hemisuccinate building block **6a** (see Basic Protocol 2)
4-Dimethylaminopyridine (DMAP)
N,*N*-Diisopropylethylamine, redistilled (see recipe)
Acetonitrile (MeCN) anhydrous (see recipe)
O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU; **13**)
Aminopropyl- or LCAA CPG or Aminomethyl Macroporous Polystyrene
Anhydrous pyridine (see recipe)
N-Methylimidazole
Acetic anhydride, reagent grade
Methanol, reagent grade
Ethyl acetate, reagent grade

Round-bottom flasks
Shaker, wrist action or orbital
Pasteur pipet
Sintered glass filter
Vacuum desiccator
Oil or diaphragm pump
Bunsen flask
Whatman no. 1 filter paper
Buchner funnel
Screw-capped glass vials

Additional reagents and equipment for trityl analysis (see Support Protocol 3) and Kaiser test (see Support Protocol 4)

CAUTION: Coupling agent TBTU can cause severe skin or eye irritation and allergic reactions. Use appropriate skin and eye protection, and wipe down all work areas and utensils with a wet cloth immediately after use (TBTU is water soluble).

Couple hemisuccinate of the building block to aminopropyl CPG

1. Calculate the scale of loading and the consumption of reagents. In this protocol, 100 g of aminopropyl CPG1000 is being loaded with the triethylammonium hemisuccinate of a mono-DMT-protected diol to 45 to 50 μmol/g. Thus, the scale of loading is ∼5.0 mmol.

Activate hemisuccinate of the linker

2. In a 250-mL flask equipped with a stopper, dissolve the hemisuccinate building block **6a** (1.10 eq., 5.5 mmol) and DMAP (0.1 eq., 61 mg) in anhydrous acetonitrile (50 mL) and anhydrous *N*,*N*-diisopropylethylamine (3 eq., 1.93 g, 2.6 mL). Add TBTU (1.15 eq., 1.85 g) in one portion, close the flask, and stir the mixture until TBTU dissolves completely. Set the flask aside for 10 to 15 min.

3. In a 2-L round-bottom flask, suspend 101 g aminopropyl CPG1000 in 400 mL anhydrous acetonitrile.

4. Transfer the activation mixture (see step 2) to the flask containing CPG (from step 3). Rinse the activation flask with 10 mL anhydrous acetonitrile and transfer the liquid to the flask containing CPG. Close the flask securely and shake in a wrist-action or orbital shaker for 4 hr.

   The rate of shaking should be sufficient to maintain the solid phase suspended. The overhead stirrer may also be used in a manner described above for preparation of aminopropyl CPG. On scales exceeding 250 g CPG, the use of the overhead stirrer is recommended.

5. Check the course of the coupling reaction by collecting a small sample of support (∼20 to 50 mg) with a Pasteur pipet and applying it to a sintered glass filter. Wash the solid support with 5% triethylamine in acetonitrile (3×5 mL) and acetonitrile (3×5 mL). Place the funnel in a vacuum desiccator and dry on an oil or diaphragm pump at room temperature for ∼15 min. Determine the loading by trityl analysis (see Support Protocol 3).

6. In rare cases when the loading is insufficient, calculate the amount of succinate to compensate for the balance. Activate the succinate using the ratios of reagents given above and add the activated mixture to the suspension of CPG.

Cap the unreacted amino groups

7. Once the desired loading is obtained, add pyridine, *N*-methylimidazole, and, finally, acetic anhydride (10 mL, 5 mL, and 5 mL, respectively, for every 100 g of CPG) to the same flask. Seal the flask and shake for 1 hr at room temperature.

8. Check the course of the capping reaction by collecting a small sample of support (∼20 mg) with a Pasteur pipet and applying it to a sintered glass filter. Wash the solid support with acetonitrile (3×5 mL), and conduct Kaiser test (see Support Protocol 4).

9. If the Kaiser test is positive (some amino groups remain uncapped as manifested by a blue color), shake the mixture for another hour and check for completion again. If that occurs, use fresh lots of capping agents for your next capping reaction.

   Do not run the capping reaction longer than for 2 hr. This may lead to an extensive loss of loading.

10. Using a Bunsen flask equipped with a 1-L sintered glass filter or Buchner funnel with Whatman no. 1 filter paper, filter off the support and wash with methanol (3×400 mL) and then with ethyl acetate (2×400 mL). Dry the support in a vacuum oven at 35°C or on rotary evaporator using an adapter equipped with a sintered glass frit.

11. Determine the loading by trityl analysis of a small portion of the final product (see Support Protocol 3).

Store 4 to 6 weeks supply at room temperature in a sealed screw-capped vial. A long-term supply should be stored at −18°C under anhydrous conditions. Supports can be stored for at least 2 years.

EXPEDITE ATTACHMENT OF HEMISUCCINATES OF DMT-PROTECTED BUILDING BLOCKS TO AMINOPROPYL CPG

ALTERNATE PROTOCOL

The protocol below describes the expedite loading of aminopropyl CPG on a 10-g scale. The preparation may be scaled down to 1 g without any changes in molar ratios of the reagents. On a small scale (100 mg to 1 g), it is advisable to use 2.5 to 3 eq. of the activated succinate with respect to the desired loading. However, we do not recommend using an excess larger than 2.1 eq. on the scale of 1 g and larger. Under these conditions, the loading would proceed too rapidly to be controlled, and the chance of obtaining an overloaded support is very high. The protocol is designed for the time-efficient operation to obtain solid supports loaded on par or slightly over the specification. For materials, see Basic Protocol 3.

Couple hemisuccinate of the building block to aminopropyl CPG

1. Calculate the scale of loading and the consumption of reagents based on the amount of solid support needed and its loading.

For example, in this general protocol, 10 g of aminopropyl CPG1000 is being loaded with the triethylammonium hemisuccinate of a mono-DMT-protected diol to 45 to 50 μmol/g. Thus, the scale of loading is 0.5 mmol.

Activate hemisuccinate of the linker

2. In a 10- to 25-mL flask equipped with a stopper, dissolve the hemisuccinate of the linker (2.10 eq., 1.05 mmol), and DMAP (0.21 eq., 13 mg) in anhydrous acetonitrile (5 mL) and anhydrous *N*,*N*-diisopropylethylamine (6.3 eq., 0.41 g, 0.55 mL). Add TBTU (2.15 eq., 0.346 g) in one portion, close the flask, and stir the mixture until TBTU dissolves completely. Set the flask aside for 10 to 15 min.

3. In a round-bottom flask, suspend 10.5 g aminopropyl CPG1000 in 40 mL anhydrous acetonitrile.

4. Transfer the activation mixture to the flask containing CPG. Rinse the activation flask with 10 mL anhydrous acetonitrile and transfer the liquid to the flask containing CPG. Close the flask securely and shake in a wrist-action or orbital shaker for 45 min.

The rate of shaking should be sufficient to maintain the solid phase suspended.

While the solid support is being loaded, prepare solutions and glassware for determination of the loading by DMT test (see Support Protocol 2).

5. Check the course of the coupling reaction by collecting a small sample of support (∼20 to 50 mg) with a Pasteur pipet and applying it to a sintered glass filter. Wash the solid support with 5% triethylamine in acetonitrile (3×5 mL) and acetonitrile (3×5 mL). Place the funnel in a vacuum desiccator and dry on an oil or diaphragm pump at room temperature for ∼5 min. Determine the nucleoside loading by trityl analysis (see Support Protocol 3).

With experience, the total time required for DMT determination does not exceed 15 min.

6. In rare cases when the loading is insufficient, make your judgment; if the loading was lower by ∼5 μmol/g, it is very likely that the support got loaded to the specs over the time consumed by the determination of DMT. If the loading is sufficiently lower than required, calculate the amount of succinate to compensate for the balance.

Activate the succinate using the ratios of reagents given above and add the activated mixture to the suspension of CPG. Prior to further loadings, check the quality of all reagents.

Cap the unreacted amino groups

7. Start capping immediately after the satisfactory loading was proven. Follow steps 5 to 9 from Basic Protocol 1.

SUPPORT PROTOCOL 1

DETERMINATION OF SOLID SUPPORT-BOUND AMINO GROUPS BY PICRIC ACID TEST

This procedure describes the determination of the surface loading of CPG supports containing amino groups by colorimetric analysis (adapted with minor modifications from Houghten et al., 1986). The procedure uses binding of picric acid to the amino groups followed by elution of the bound material with an ethanolic base. Picric acid and its solution display a bright yellow color, which is used for spectrophotometric quantification.

Materials

Picric acid
Dichloromethane, reagent grade
Anhydrous magnesium sulfate
Aminoalkyl solid support
N,*N*-diisopropylethylamine (see recipe)
10% and 95% Ethanol (EtOH)

Tightly capped flasks
Disposable glass pipets with wool plugs
10-mL volumetric flasks
Quartz cuvettes
UV/VIS spectrophotometer or colorimeter

1. Prepare a 0.05 M solution of picric acid in 20 to 50 mL CH_2Cl_2. Dry the solution with anhydrous magnesium sulfate. Using a Bunsen flask equipped with a 20-mL sintered glass filter or Buchner funnel with Whatman no. 1 filter paper, filter the drying agent off. Store the obtained solution in a tightly capped flask.

2. Weigh aliquots (~5 to 10 mg) of oven-dried aminoalkyl solid support in disposable glass pipets with glass wool plugs.

3. Wash the solid support with 5% *N*,*N*-diisopropylethylamine in CH_2Cl_2 (3 × 1 mL), 0.05 M picric acid in CH_2Cl_2 (4 × 1 mL), and finally with 10% EtOH in CH_2Cl_2 (4 × 1 mL).

4. Elute picric acid from samples with 5% *N*,*N*-diisopropylethylamine in 95% EtOH (9 to 10 mL) collecting the eluate in a 10-mL volumetric flask. Fill up the flask with 5% *N*,*N*-diisopropylethylamine in 95% EtOH to the graduation ring, insert a stopper, and mix well.

   The loading with amino groups depends on the specific surface area and hence on the pore size of aminopropyl CPG. For CPG500, samples of ~5 mg should be taken, and the solution should be additionally diluted with 95% EtOH at least twice. For samples of aminopropyl CPG1000 taken in the amount of ~5 mg, the dilution is normally not required, while for wider-porous CPG, 10 mg sample with no dilution is recommended.

5. Measure the optical density of the solution in a quartz cuvette with a path length of 1 cm at $\lambda = 358$ nm using 95% EtOH as a reference sample.

6. Calculate the loading with amino groups using the weight of the sample, the volume, the dilution factor, the optical density, and the extinction coefficient of 14,500 at $\lambda = 358$ nm according to the following equation:

$$\text{Loading}\left(\frac{\mu\text{mol}}{\text{g}}\right) = 1000 \times \frac{\text{Volume (mL)} \times \text{Dilution} \times \text{Absorbance}}{14.5 \times \text{Weight (mg)}}$$

For undiluted samples of 10 mL, the equation is reduced to the following:

$$\text{Loading}\left(\frac{\mu\text{mol}}{\text{g}}\right) = 690 \times \frac{\text{Absorbance}}{\text{Weight (mg)}}$$

DETERMINATION OF AMINO GROUPS BY DMT TEST

SUPPORT PROTOCOL 2

An alternative to Support Protocol 1, the method presented below uses the dimethoxytrityl chloride/tetrabutylammonium perchlorate reagent developed for the solid-phase tritylation of nucleosides and nucleotides (Reddy et al., 1987). This reagent will also tritylate amino groups, and it has been used to monitor coupling efficiency in solid-phase peptide synthesis (Reddy and Voelker, 1988). This is a very fast, sensitive, and general method for determining the number of amino and hydroxyl groups on insoluble supports.

Materials

Aminoalkyl-derivatized solid support
0.25 M 4, 4′-dimethoxytrityl chloride stock solution (see recipe)
0.25 M tetrabutylammonium perchlorate stock solution (see recipe)
Methanol, reagent grade
Dichloromethane, reagent grade
Screw-capped glass vials

1. Place ~10 to 20 mg of amino-derivatized CPG support in a small screw-capped glass vial and add equal amounts (~500 μL) of 0.25 M 4,4′-dimethoxytrityl chloride and 0.25 M tetrabutylammonium perchlorate. Seal the vial and shake 10 min at room temperature.
2. Using a Bunsen flask equipped with a 10-mL sintered glass filter, filter off the support, and wash first with 10 to 20 mL dichloromethane, then with 10 to 20 mL methanol, and finally with 20 to 30 mL dichloromethane again.

   The methanol wash is important to remove any adsorbed trityl reagent from the support.

3. Allow the support to dry and then perform the colorimetric trityl analysis (see Support Protocol 3).

QUANTITATIVE DETERMINATION OF SURFACE LOADING OF DERIVATED SUPPORTS BY TRITYL ASSAY

SUPPORT PROTOCOL 3

This procedure describes the determination of the surface loading of supports containing trityl groups by colorimetric analysis. The method is based on the ability of mono-, di-, and trimethoxytrityl-protecting groups to cleave under acidic conditions releasing in the solution brightly colored respective trityl cations. In the absence of quenchers (any nucleophiles), the color remains stable. Its intensity and hence the concentration of the

chromophore, the trityl cation, can be measured spectrophotometrically. As the amount of the cation equals the amount of the mono-, di-, or trimethoxytrityl-protected linker attached to the solid support, the loading can be directly calculated from the absorbance of the acidic test solutions.

Materials

Derivatized aminopropyl CPG support (see Basic Protocol 3 and Alternate Protocol)
Detritylation reagent (see recipe for TFA solution)

10-mL volumetric flask
Analytical balance accurate to at least 0.1 mg
Quartz cuvettes
UV/VIS spectrophotometer or colorimeter

1. Tare an empty 10-mL volumetric flask on an analytical balance.
2. Weigh dry, derivatized aminopropyl CPG support (~5 to 7 mg) directly into the flask. Record the weight to at least 2 significant figures.
3. Add solution of TFA (see recipe for detritylation reagent) to the volumetric flask to the mark. Seal the flask and mix.

   An orange-colored dimethoxytrityl cation (yellow and red for mono- and trimethoxytrityl cations, respectively) will immediately form.

   The color of trityl cations is stable in the absence of nucleophiles (moisture, alcohols, etc.). The use of an anhydrous solution of TFA is recommended to prevent underestimated DMT readings. Dichloromethane, the solvent used in the test, evaporates relatively readily from open containers thus reducing the accuracy of the analysis. The evaporation of the samples should be minimized by closing the volumetric flask and the filled cuvettes.
4. Dilute the sample 10 times with solution of TFA, mix the diluted sample.
5. Fill a reference cuvette with the solution of TFA or, if using a single-beam spectrophotometer, run a baseline.

   Disposable polystyrene cuvettes are incompatible with the use of dichloromethane as a solvent. Only quartz cuvettes can be used.
6. Record the spectrum of the sample from 400 to 600 nm. Measure the absorbance of the peak maximum at 486 nm (trimethoxytrityl group), ~503 to 505 nm (dimethoxytrityl group), or ~470 nm (monomethoxytrityl group).

   With the suggested amount of sample and dilution, the solid supports loaded in a range from 25 to 190 μmol/g will give the absorbance readings from 0.1 to 1 absorbance units, well within the linear range of most spectrophotometers. If the anticipated loadings are outside of that range, the dilution factor should be modified accordingly.
7. Calculate the loading of the support using the following equation (for a cuvette with a 1 cm path length):

$$\text{Loading}\left(\frac{\mu\text{mol}}{\text{g}}\right) = \frac{\text{Volume (mL)} \times \text{Dilution} \times \text{Absorbance}}{\varepsilon \times \text{Weight (mg)}}$$

where ε is the extinction coefficient of the trimethoxy, the dimethoxy, or the monomethoxytrityl group (116, 76, and 56 mL cm^{-1} μmol^{-1}, respectively).

In the case of 4,4′-dimethoxytrityl cation, the suggested sample volume of 10 mL and the dilution factor of 10, the equation above is reduced to the following:

$$\text{Loading}\left(\frac{\mu\text{mol}}{\text{g}}\right) = 1316 \times \frac{\text{Absorbance}}{\text{Weight (mg)}}$$

DETECTION OF AMINO GROUPS ON INSOLUBLE SUPPORTS BY KAISER TEST

SUPPORT PROTOCOL 4

The following protocol describes the detection of solid support-bound amino groups remaining after a solid support was loaded with a linker and capped. The protocol is based on the work by Kaiser et al. (1970). The protocol uses a reaction of ninhydrin (**14**, Fig. 3.2.4) with the primary amino groups, which results in the formation of Ruhemann's Blue, **15**, a dark-blue dye (Sarin et al., 1981). The preparation of the solutions required is described below. A three-component formulation performing the same function can be obtained from Fluka as Kaiser test kit (cat. no. 60017).

Additional Materials *(also see Basic Protocol 1)*

Ninhydrin, reagent-grade
Phenol, reagent-grade
Potassium cyanide (KCN), reagent-grade
Acetonitrile (MeCN)

Heating block
Vortex mixer
Microcentrifuge

CAUTION: KCN is highly toxic. Ingestion of solid or solutions or inhalation of dust may result in death. Wear gloves and eye protection, and conduct any work with KCN in a fume hood.

1. Prepare Reagent A by combining the following:

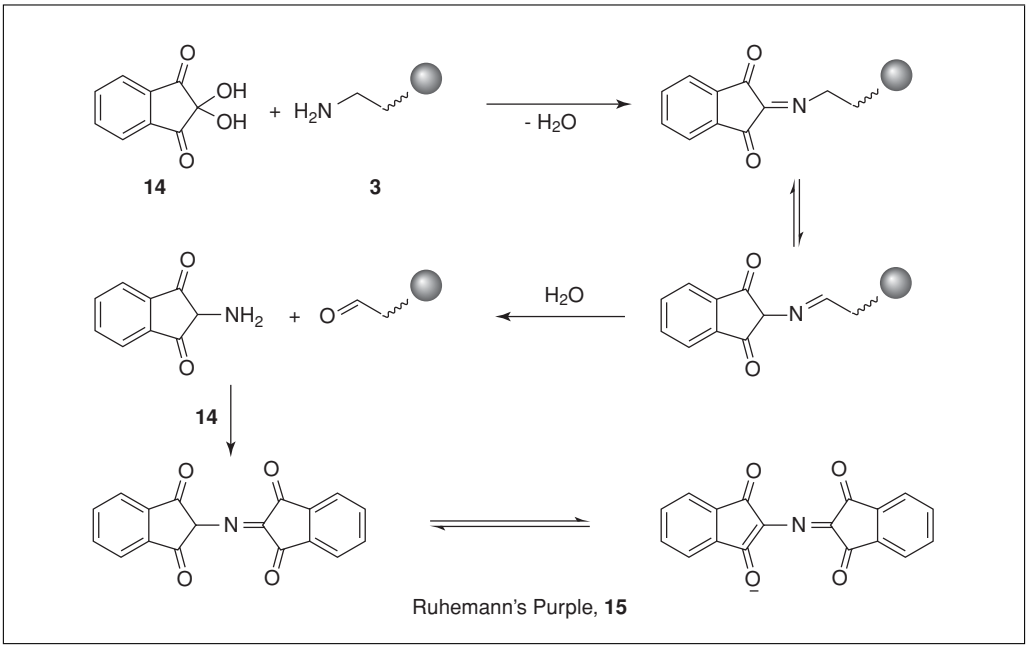

Figure 3.2.4 Reaction of ninhydrin, **14**, with aminoalkyl-derivatized solid support to form Ruhemann's Purple, **15**, a dark-blue colored dye.

40 g phenol
98 mL pyridine
10 mL 95% ethanol
1.3 mg KCN
2 mL water.

It is critical to observe the correct concentration of KCN in the reagent because an excessive amount results in a side reaction leading to the formation of red-brown color in experimental and blank samples. For best result, prepare 0.01 M aqueous KCN (about 10 mL). Add 2 mL of aqueous KCN obtained to the mixture of phenol, pyridine, and ethanol. For a safe disposal of the remaining aqueous KCN, release the used solutions, under a ventilated fume hood, in an excess of 20% to 30% aqueous hydrogen peroxide and leave the mixture overnight.

2. Prepare Reagent B by combining the following:

   2.5 g ninhydrin
   50 mL of 95% ethanol.

   Reagents A and B may be stored for several years in screw-cap vials at room temperature.

3. Switch heating block on and set the temperature to 105°C.

4. Prepare the test sample and controls.

   Using a Pasteur pipet, collect a small sample of loaded and capped CPG in a 1.5- to 2-mL plastic test tube. Add 1 mL MeCN, vortex the test tube, spin down, and withdraw the solution. For a convenient observation, the amount of the solid support should be sufficient to form a layer of 2 to 3 mm on the bottom of the test tube.

   As reference samples, use approximately same amounts of aminopropyl CPG (positive control) and native CPG (negative control) placed in separate test tubes.

5. Perform the test as follows:

   a. Add 50 µL of each Reagent A and B to the test sample and the control samples.
   b. Vortex and place the test tubes in the heating block for 3 min.

6. Read the results.

   Withdraw the test tubes and immediately observe the color formed (the color fades over a short period of time).

   The solid support in the negative control (native CPG) remains colorless, and the solution stays yellow.

   The solid support and the liquid phase in the positive control (aminopropyl CPG) are of dark-blue color.

   A well-capped solid support should match the negative control. Samples with insufficient capping may show various shades of blue.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetonitrile, anhydrous

Distill acetonitrile (MeCN) from calcium hydride or obtain commercially available anhydrous solvent. Acetonitrile of "low-water" grade currently offered by J. T. Baker (cat. no. 9018-03, max 10 ppm), BDH (cat. no. PL017ZA-4, max 30 ppm), and Honeywell (Burdick & Jackson, cat. no. NP017-10, max 30 ppm) is quite suitable for loading of solid supports. Store over molecular sieves in tightly sealed bottles.

Controlled-pore glass (CPG)

Native, aminopropyl, and long chain alkylamine CPG is commercially available from Biosearch, Integrated DNA Technologies, Millipore, and Prime Synthesis in a variety of pore and particle sizes and sieved into three sets of particle sizes: 80 to 120 mesh (125 to 177 μm), 120 to 200 mesh (74 to 125 μm), and 200 to 400 mesh (37 to 74 μm); the two largest size ranges are the most suitable for oligonucleotide synthesis. The material can be stored safely at room temperature for >5 years.

The same manufacturers also supply all nucleoside CPG with the standard and the base-labile protecting groups. Most recently, nucleosidic CPG is being replaced with universal CPG, various versions of which are available from AM Chemicals ChemGenes, Glen Research, and Metkinen. Finally, a wide variety of standard loaded CPG and solid supports for special applications is available from Glen Research.

Detritylation reagent

Trifluoroacetic acid (TFA) in anhydrous dichloromethane (3%, v/v) is recommended as an all-purpose acidic solution for determination of DMT groups bound to solid supports. Dichloroacetic acid (3% v/v) or trichloroacetic acid (2%, w/v) in dichloromethane or 1,2-dichloroethane may be used for most determinations, but lack general applicability. Store indefinitely at room temperature.

CAUTION: *The use of dichloromethane of grades lower than anhydrous may lead to underestimated readings due to the quenching of trityl cations by water. The use of anhydrous dichloromethane is strongly recommended.*

CAUTION: *Solution of TFA should not be used as a detritylation reagent in oligonucleotide synthesis where 3% (v/v) dichloroacetic acid or 2% (w/v) trichloroacetic acid in dichloromethane (sometimes referred to as "deblock" reagent) are recommended.*

CAUTION: *Many older references suggest the use of perchloric acid solutions to dilute the trityl colors. Such a strong acid is not required, and for safety reasons, perchloric acid solutions should not be used. The same detritylation reagent used on the DNA synthesizers can be used to dilute the colors for measurement.*

Diisopropylethylamine (DIEA), anhydrous

Purchase as the 99.5% redistilled grade (Aldrich), and keep anhydrous by storage over type 4 Å molecular sieves. Store in septum-sealed bottles for 1 year at room temperature, protected from light.

Dimethoxytrityl chloride stock solution, 0.25 M

Dissolve 847 mg (2.5 mmol) 4,4′-dimethoxytrityl chloride in 10 mL dichloromethane. Store in a sealed glass vial up to 3 to 4 weeks at room temperature.

N-protected 5′-O-DMT nucleosides

If the objective is the preparation of nucleosidic solid supports, purchase 5′-*O*-dimethoxytrityl nucleosides with a free 3′-OH or, for ribonucleosides, free 2′- and 3′-OH groups (ChemGenes, Chem-Impex, Rasayan, or Sigma). N^6-benzoyl-5′-*O*-dimethoxytrityl-2′-deoxyadenosine, N^4-benzoyl-5′-*O*-dimethoxytrityl-2′-deoxycytidine, N^2-isobutyryl-5′-*O*-dimethoxytrityl-2′-deoxyguanosine, and 5′-*O*-dimethoxytritylthymidine are the most commonly used standard nucleosides and are compatible with the protocols described in this unit. Store up to 5 years at −20°C.

Pyridine, anhydrous

Prepare by distillation from calcium hydride, and keep anhydrous by storage over type 4 Å molecular sieves in tightly sealed bottles. Store up to 1 year at room temperature.

Alternatively, anhydrous pyridine may be purchased ready to use in Sure/Seal bottles (Aldrich). Pyridine graded "low water" (Honeywell) is acceptable.

Tetrabutylammonium perchlorate stock solution, 0.25 M

Dissolve 854 mg (2.5 mmol) tetrabutylammonium perchlorate (Sigma) in 0.3 mL 2,6-lutidine and 9.7 mL dichloromethane. Store in a sealed glass vial up to 3 to 4 weeks at room temperature.

COMMENTARY

Background Information

The surface of CPG presents only silanol functions that are not useful for the direct attachment of linkers or phosphoramidite building blocks required to serve as anchoring points for oligonucleotide chain assembly. The most user-friendly and widely accepted approach for derivatization of the surface of CPG **1** is shown in Fig. 3.2.1 and consists in the treatment of silanol surface with 3-(triethoxysilyl)-1-propanamine **2**, which derivatizes the surface with primary aliphatic amino groups. The obtained aminopropyl CPG **3** can be conveniently used for the attachment of linkers or for further conversion to LCAA CPG. As follows from its name, macroporous aminomethyl polystyrene for oligonucleotide synthesis already bears amino groups on the surface and hence does not require any additional treatment before the attachment of linkers.

The first building block is often attached to the solid phase via an additional linker arm. The succinic acid linker was one of the earliest linkages used to immobilize nucleosides to insoluble supports (Ogilvie and Kroeker, 1971; Yip and Tsou, 1971), and it has remained widely used since. Although a variety of different linkages have been developed for specialized applications, routine oligonucleotide synthesis has been well served by the succinyl linker. Succinic anhydride **5a** (Fig. 3.2.2) is an inexpensive starting material that does not require any additional activation to be reacted with alcohols. The reaction of succinic anhydride **5a** with an alcohol **4** results in the homosuccinate ester **6a** bearing the terminal carboxylic acid group, which makes protected nucleoside-3′-*O*-succinyl hemiester starting materials **7** and **8**, hemisuccinate of universal linker **9**, or any other hemisuccinate building blocks readily available. Yet, the succinate ester function is hydrolyzed or aminolyzed under moderately mild conditions with the rate of cleavage exceeding or matching that of the most widely used nucleic base protecting groups. This makes the succinyl arm suitable to fulfill the demands of routine synthesis of oligonucleotides with DNA and RNA backbones and their phosphorothioate analogs. Replacement of **5a** with diglycolic anhydride **5b** gives diglycolate hemiesters **6b** without any modifications to the synthetic protocol (Pon and Yu, 1997a).

Generally, a solid support of formula **12** may be obtained by two methods (methods A and B in Fig. 3.2.3). In the historically first method A, a hemisuccinate **6a** is prepared in solution and is coupled to aminopropyl- or LCAA CPG **3** to form the desired solid support **12a**. The alternative method B uses succinylation of aminopropyl or LCAA CPG with succinic anhydride to give the succinylated CPG **14a**. After capping with acetic anhydride, **14a** is treated with the linker **4** having a reactive hydroxy group in the presence of 1-ethyl-3-[3-(*N*,*N*-dimethylamino)propyl]carbodiimide (DEC, EDAC, or EDC) to give **12a** (Damha et al., 1990; Pon et al., 1988; also see *UNIT 3.13*). It is important to understand that both methods have their advantages and their weak points. The disadvantage of method A is that it requires an additional synthetic step of preparation of a hemisuccinate **6a**, possibly complicated by its purification by a silica gel column chromatography. However, frequently used hemisuccinates can be prepared on large scale and kept in stock. Once **6a** is available, the coupling of it to the aminopropyl CPG 3 requires a very small excess of **6a** and allows a reasonably accurate control of the loading of the solid support **12a**. In contrast, method B does not require the preparation

of **6a**. However, a several-fold excess of the linker **4** is required for the successful coupling reaction with **14a**, the coupling itself takes longer unless the reaction is carried out in a microwave oven as described in *UNIT 3.13*, a more extensive in-process control is necessary to obtain the loading accurately falling in the desired range, and the consumption of organic solvents is several times greater.

When a solid support is prepared on a small scale for internal use, these advantages and disadvantages, perhaps, cancel each other so that it does not really matter which method to use. We recommend that those unfamiliar with preparation of solid supports test both methods to identify the one that best fits the specific requirements, work environment, and personal preferences. Method A is described in this unit while method B has been described in great detail in the past (Damha et al., 1990; Pon et al., 1988; also see *UNIT 3.13*).

When solid supports are prepared on a large scale, the choice of the method becomes more critical and more cost-dependent. In our experience, the most time-consuming, laborious, and expensive step in preparations on a large scale is washing the solid supports. To make this point clearer, let us consider some physical properties of the most frequently used CPG500 with the typical particle size of 120 to 200 mesh (75 to 125 μm). Porosity (the ratio of a void volume to the bulk volume of material) and pore volume of bulk CPG500 are ~85% and 1.2 to 1.3 mL/g, respectively. Bearing in mind that the density of solid SiO_2 is 2.2 g/mL, one deduces that the volume inside the pores accounts for about 40% of the total void volume. When a bulk of CPG is allowed to absorb that much of a nonviscous solvent, e.g., acetonitrile, the volume fraction of absorbed acetonitrile will equal the porosity, or ~0.85 of the bulk volume. At the end of the process, the total volume of the bulk material will not change. At this point, the CPG will be tightly packed, so if one wanted to make the material reasonably mobile, for instance, to stir it on a sintered glass filter, one would need to add more solvent. A volume that allows convenient mixing on the filter would be ~120% to 125% of the bulk volume of CPG. Once the mixing is finished, the solvent is filtered off. Importantly, the portion contained in the pores will not be removed readily by filtration. Additionally, some amount of the solvent will be retained in the bulk of the material by capillary forces so that a volume of the solvent equal to ~50% of that of the bulk will remain in CPG unfiltered.

With these figures in hand, let us consider the scenario where the void volume of a bulk CPG is filled with acetonitrile containing a chemical in an amount of 1 arbitrary unit. The objective is to reduce the contents of the chemical to <0.01% of the initial amount by the successive filtration and washing with a fresh solvent. The initial filtration will remove 0.5/0.85≈59% of the chemical. A fresh solvent (125% of the bulk volume) is then added; the mixture is stirred and filtered again. A simple calculation shows that a total of five additions of a fresh solvent and six filtrations are required to meet the specs at the expense of 6.25 vol of a solvent. Adding ~1.5 vol of a solvent to carry out a reaction with CPG, one deduces that one chemical step consumes at least 7.75 vol of solvents per vol of CPG or 24.2 L per kg of CPG. The lesson from these figures is that, on a large scale, the most advantageous and economical method is the one with the least number of synthetic steps conducted on solid phase. In the specific case considered here, we recommend the use of method A, where only two steps, amination and loading, are carried out on solid phase.

The use of method A requires an efficient coupling of hemisuccinates **6a** and **6b** to the support-bound amino groups. Under mild conditions, the coupling reaction of an amine (i.e., aminopropyl CPG) to the carboxylic group occurs only after conversion of the carboxylic group to its activated derivatives. Of these, an acid chloride (Ogilvie and Kroeker, 1971; Sharma et al., 1992) and a symmetrical anhydride (Gait et al., 1980) have been reported. Esters activated by coupling to acidic phenols, namely pentachlorophenyl (Miyoshi et al., 1980), *p*-nitrophenyl (Koster et al., 1983), or pentafluorophenyl derivatives (Efimov et al., 1993) have been widely used. These intermediates, however, require an additional coupling step to prepare, are hydrolytically labile, and the support derivatization reactions are slow. A carbodiimide method using dicyclohexylcarbodiimide (DCC; Chow et al., 1981; Matteucci and Caruthers, 1981; Montserrat et al., 1993) or, more conveniently, DEC (Pon et al., 1988), has also been popular.

More recently, improved coupling methods have been described using an oxidation-reduction coupling reagent combination, triphenylphosphine and 2,2′-dithiobis(5-nitropyridine). Under these conditions and at the expense of a large excess of nucleoside

hemisuccinates, the coupling time was reduced to between 2 and 30 min (Gupta et al., 1995; Kumar et al., 1996). Use of diisopropylcarbodiimide and hydroxybenzotriazole (Bhongle and Tang, 1995) or DCC and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Walsh et al., 1997) allows satisfactory derivatization of aminopropyl- and LCAA-CPG supports. Extremely efficient derivatization is obtained by using a combination of uronium- or phosphonium-coupling reagents and DMAP. With a large excess of nucleoside succinates and coupling agents, the loading may be completed in only 4 sec (Pon and Yu, 1997b). The protocols in this unit describe a more conservative version of this method that uses TBTU (**13** in Fig. 3.2.3; Dourtoglou et al., 1984) as a coupling agent and allows a precise control of the loading.

Recently, the development of novel methods in molecular biology has created a great demand for large numbers of synthetic oligonucleotides, and a number of productivity enhancements have been implemented in oligonucleotide synthesis. Notably, new high-throughput DNA synthesizers operating in multi-well plate format have been developed. When four nucleosidic solid supports were used in multi-well plate format, each plate had to have its own, order-specific configuration of packing. This proved to be a time consuming and an error-prone step that could not be performed in advance. A significant increase in productivity has been achieved by using universal solid supports developed over the past 10 years. The protocols provided are well compatible with universal linkers of hexahydro-5,6-dihydroxy-4,7-epoxy-1*H*-isoindole-1,3(2H)-dione family (**9** in Fig. 3.2.2; Guzaev and Manoharan, 2003; Kumar et al., 2006; Ravikumar et al., 2008).

Critical Parameters and Troubleshooting

Handling of the support

CPG-based solid supports are rigid and mechanically fragile. They must be handled carefully to prevent particle fracturing and the formation of fines because these small particles can plug filters or create higher back pressures in synthesis columns. It is imperative to avoid the use of magnetic stirrers when performing coupling reactions. However, mechanical overhead stirrers are quite suitable and are the preferred choice on a large scale. Orbital or wrist-action shakers are the preferred method for small scale preparations.

Quality reagents and solvents

Lower-than-expected support loadings are obtained if the coupling reagents (TBTU, HBTU, or similar) have been exposed to moisture contamination. These reagents should be kept at –20°C for long-term storage and not opened until warmed to room temperature to prevent contamination from moisture condensation. A more common problem is the presence of moisture in the anhydrous reactions. Both pyridine and acetonitrile are quite hygroscopic, and storage over molecular sieves is strongly recommended. One of the main sources of water contamination is aminopropyl CPG, a relatively hygroscopic material whose extensive drying in vacuum at elevated temperature is strongly recommended. Although TBTU-activated carboxylic acids have a fairly high selectivity towards amino groups, excessive moisture will cause poor results.

Characterization and quality control of solid supports

Direct characterization of products attached to the surface of insoluble supports is very difficult, if not impossible. This is especially so with a rigid, nonswelling, inorganic support such as controlled-pore glass beads. Therefore, the success of loading reactions is usually determined indirectly by examining cleavage products. This can range from the comprehensive characterization of nucleoside or oligonucleotide products hydrolyzed from the support to the simple trityl analysis described in this unit. The 4,4′-dimethoxytrityl protecting group, commonly used to block the 5′-OH of nucleosides and hydroxy groups of other building blocks, provides a convenient and highly sensitive marker (Support Protocol 2). There is a 1:1 relationship between the amount of orange-colored dimethoxytrityl cation released and the amount of the linker attached, so the loading of any support can be easily determined by a colorimetric measurement. Because the trityl group is easily cleaved from the support by acids and it generates such a distinctive and easily quantitated species, it is much simpler and faster to perform a trityl analysis than an analysis of the attached linker.

The trityl results can be quite accurate and reproducible, as long as care is taken to avoid the following three possibilities. First, the trityl colors are most stable in the presence of excess acid. Therefore, dilutions should be performed by adding acidic deprotection reagent and not plain solvent. Second, the orange color is quenched by traces of protic solvents, such as water or alcohols. Thus, glassware and

cuvettes should be clean and dry before use. Finally, the trityl assay does not distinguish between building blocks (or trityl groups) that are covalently and noncovalently bound. Thus, it is possible for supports that have not been adequately washed to give false trityl results. This is not usually a problem, because most unbound reagents are easily removed by an acetonitrile wash. Similarly to any other anion-exchange matrix, in the absence of excess strong base, amino supports bind negatively charged hemisuccinates strongly enough to resist washing. To avoid artificially elevated results of DMT determination, the uncapped solid supports should be washed with 5% TEA in acetonitrile. The loading should also be tested again after acetylation of the surface amino groups (i.e., the capping step with acetic anhydride/NMI/ pyridine).

The number of functional groups on the surface of the support can also be determined by trityl analysis, if they can be derivatized with trityl groups. Amino and/or hydroxyl surface loadings are easily determined through the dimethoxytrityl derivatization procedure described in Support Protocol 2 (Reddy and Voelker, 1988).

Characterization of novel hemisuccinate building blocks should be carried out using ^{1}H and ^{13}C NMR spectroscopy plus combustion analysis or HR MS in combination with HPLC. If samples of authentic material are available, an identity test may be performed by HPLC or TLC. In-process control can be most easily performed by thin-layer chromatography (TLC). If the identity of the starting mono-DMT protected diols is well established (i.e., commercially available material), observation of the complete conversion of the starting material into a slower-moving product (hemiester) by TLC analysis, along with satisfactory trityl results from the derivatized support, are usually sufficient.

Reagent concentration

One of the most important factors in reactions involving solid-phase supports is the solution concentration, and the easiest way to improve results, without consuming excess reagents, is to increase the concentration of an activated linker by reducing the reaction volume. This method is limited, however, by the volume required to suspend the support. Typically, at least 4 mL of solution per gram of aminopropyl- or LCAA-CPG is the minimum required to maintain the solid supports in suspension, although the convenience of manipulation may require more solvent if the scale is small. Generally, the protocols described in this unit do not depend strongly on reagent concentration, and reasonable results can be obtained without careful measurement of solvent volume. Researchers should be aware, however, that excessive solvent will lower results through both dilution effects and increased moisture content (if solvents are not completely anhydrous).

TBTU side reaction with amines

Researchers should also be aware that *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU coupling reagent, **13**), originally developed for peptide synthesis (Knorr et al., 1989), can form a Schiff base with amino groups (Gausepohl et al., 1992; Story and Aldrich, 1994). Therefore, the pre-activation of the carboxylic acid with the coupling agent followed by the addition of the activated mixture to the amine is the preferred method of carrying out the coupling reaction. In no event should the coupling reagent be added to aminopropyl or LCAA-CPG in the absence of the carboxylic acid component of the coupling reaction (which normally, and very rapidly, reacts preferentially with TBTU). Thus, an excess of only 0.05 eq of TBTU over the succinate is used in Basic Protocol 3 and the Alternate Protocol to compensate for potential hydrolysis of the activated species by contaminating water. The use of a larger excess of TBTU in this reaction is neither necessary nor desirable because it results in conversion of surface amino groups to unreactive Schiff bases.

Another danger associated with TBTU stems from its ability to remove DMT-protecting group in the absence of base (Ramasamy and Averett, 1999). Although detritylation by TBTU is a rather slow process under anhydrous conditions, the coupling agent should always be added to solutions of DMT-protected succinates only in the presence of *N*,*N*-diisopropylethylamine.

Capping of unreacted amino groups on the support

An essential part of each preparation of the solid supports is the blocking (capping) of unreacted functional groups on the surface of the support. These unreacted groups are present because the amount of linkers attached is necessarily less than the total capacity of the support. In particular, it is very important to cap off any unreacted amino groups by acetylation with acetic anhydride. Otherwise, they will react with the phosphoramidite reagents

delivered during subsequent oligonucleotide synthesis cycles to produce unwanted oligonucleotide fragments with a 3′-deletion (i.e., the terminal nucleoside will be missing). These deletion fragments will be attached to the solid support via a 3′-phosphoramidate linkage and can be released from the support by either acidic hydrolysis (cleavage of the phosphoramidate bond) or prolonged ammonium hydroxide hydrolysis (cleavage of the silyl bonds joining the linker arm to the CPG surface). Although the standard cleavage and deprotection conditions (conc. aqueous ammonium hydroxide, 8 hr, 60° to 70°C) do not release significant amounts of these 3′-deletion products, they still reduce coupling yields and oligonucleotide quality by steric hindrance and competition for reagents. The effectiveness of the support capping is best verified by Kaiser test employing a reaction of ninhydrin **14** with primary amino groups (Kaiser, 1970, Fig. 3.2.4). On the other hand, the prolonged capping reduces the loading of solid supports, probably as a result of transamidation reaction. A careful process control normally allows one to complete the capping reaction without compromising the loading.

Anticipated Results

The protocols in this unit provide specific step-by-step instructions for conversion of 5′-*O*-(4,4′-dimethoxytrityl)- and base-protected nucleosides and other mono-*O*-(4,4'-dimethoxytrityl)-protected diols to their hemisuccinate esters and their coupling to CPG supports bearing aminopropyl or long chain aminoalkyl groups and to macroporous aminomethyl polystyrene (method A in Fig. 3.2.3). Additional guidelines are provided for selecting a coupling protocol and performing in-process control. The procedures described can be conducted virtually in any industrial or academic chemical laboratory. Basic training in synthetic organic chemistry should be sufficient for the proper execution of all protocols.

The protocols work for the authors on scales from grams to multi-kilogram. We certainly hope that they will work for the readers of this unit as well. Researchers with no prior experience in preparation of solid supports for oligonucleotide synthesis should be able to synthesize their first solid support without any setbacks. Those with a limited experience might find the procedures helpful for improving the accuracy of loading, batch-to-batch reproducibility, or throughput of operations. Any questions, comments, or suggestions from readers will be welcome and appreciated.

Time Considerations

The protocols in this unit describe the attachment of DMT-protected diols **4** via the succinyl linker arm and provide several procedures, some of which are optimized for minimal labor while the others provide the shortest turn-around time. In all scenarios, the most time-consuming step is the preparation of a hemisuccinate ester of a mono-DMT-protected diol, **6a**, (base-protected 5′-O-DMT nucleoside **7**, universal linker **9**, or any other). Carrying out this step requires from 1 day to 1 week depending on the protocol and on the steric hindrance around the reactive hydroxy group of a mono-DMT-protected diol.

With good planning, aminopropyl CPG requires a minimum of 24 hr for its preparation on small scale (<100 g). The shelf life for this material is unlimited. In order to avoid a last-day rush, it is advisable to maintain a stock of aminopropyl CPG of various pore sizes for the future use.

Once the hemisuccinate and the aminopropyl CPG are available, the preparation of a small amount of the loaded CPG in its finished, ready for the use in oligonucleotide synthesis form by experienced hands and at the expense of larger excess of the hemisuccinate may be carried out in about 4 hr (Alternate Protocol). With a more economical approach, a larger scale of the preparation, or a more relaxed schedule, the same procedure will require 1.5 to 2 days (Basic Protocol 3).

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