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RNA synthesis using a universal, base-stable allyl linker

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ABSTRACT

The application of a universal allyl linker, 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid, to the solid phase synthesis of RNA molecules is described. Use of this linker simplifies significantly the isolation and purification steps in RNA synthesis. The linker is universal in that it does not contain a nucleoside. The 3′ terminal nucleoside is instead attached to the support in the first coupling step. The resultant RNA fragment is then obtained as the 3′-phosphate. The linker is base-stable, and thus all reagents used during deprotection can simply be washed away, leaving the RNA attached. Further, tritylated short fragments resulting from chain cleavage for any reason are also washed away before separation from the support. This linker is compatible with any current synthetic methodology and any amino functionalized support. Of course, silica supports would not be compatible with fluoride reagents. It could also be used to advantage for other applications. Because it is cleaved under conditions orthogonal to those used during many common reactions, the range of post-synthetic manipulations that can be carried out without cleavage from the support is extended significantly.

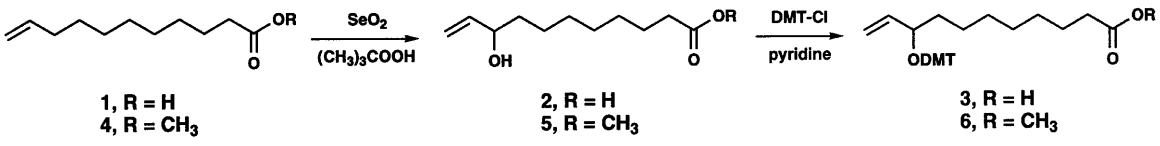
INTRODUCTION

The succinate linker commonly employed for solid-phase DNA and RNA synthesis requires preparation of specially derivatized 3′ monomers, and an additional set of reactions to load the 3′ monomer onto the support (1,2). Moreover, the lability of the succinate linkage limits the types of post-synthetic manipulations that can be carried out on the support-bound nucleic acid fragment without cleavage from the support. Several alternative linkers have been proposed recently (3–5), including our report on synthesis of 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid, as a universal allyl linker for solid-phase synthesis (6). We now describe the application of the latter linker to the synthesis of a variety of RNA molecules, as well as further details of its preparation. This linker has three distinct advantages over the conventional succinate linker. Since it is universal, no separate loading step is required for the 3′ monomer. Instead, the first coupling cycle attaches the 3′ monomer to the support, after detritylation of the linker by standard methods. Secondly, since conditions for Pd(0) mediated cleavage of the RNA fragment from the linker are orthogonal to all of the standard deprotection conditions, as well as many other possible post-synthetic transformations, the RNA can be left attached to the support throughout deprotection. This feature is particularly valuable in RNA synthesis because of the need for desilylation using, most commonly, either TBAF (tetrabutyl ammonium fluoride) (7) or triethylamine tris(hydrogen fluoride) (8,9). Finally, any trityl-containing short fragments that may be present as the result of chain cleavage during deprotection are readily removed (10).

RESULTS AND DISCUSSION

Preparation of the linker molecule, 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid (**3**), shown in Scheme 1, uses a selenium dioxide oxidation (11) of 10-undecenoic acid (**1**), or its methyl ester (**4**), to give the corresponding allylic alkoxy derivatives **2** or **5** in ~75% yield. The ester (**5**) is purified by distillation, while crude **2** is used directly in the next step. Tritylation of **2** or **5** under standard conditions using 4,4′-dimethoxytrityl chloride in pyridine gives the DMT derivatives **3** or **6**. The allyl linker molecule **3**, after purification by silica gel chromatography, can then be attached to any amino functionalized support by a variety of standard procedures (1). We use amino functionalized polystyrene/polyethylene glycol (12,13) in methylene chloride with DCC (14), which gives loadings of ~150 µmol/g, as determined by trityl assay. This allyl linker is compatible with any of the standard nucleic acid synthetic methods. We have used a hydrogen phosphonate procedure in which no amino protection is required for adenosine and guanosine (15), and cytidine is protected with a benzoyl group. It is likely that the amidite procedure could be used equally well. Further, the 5′-dimethoxytrityl group can either be removed or left on as a purification handle.

Cleavage of the product from the support is effected using 0.5–1 equiv. tetrakis(triphenylphosphine)palladium and *n*-butylammonium formate at 60°C for 2 h in a 5:1 mixture of THF and pyridine. The pyridine allows use of a slight excess of formic acid to ensure that there is no free *n*-butylamine, without risking detritylation. These mild conditions are possible because of the reactive terminal double bond in this linker. Moreover, it is likely



1, R = H
4, R = CH_3

2, R = H
5, R = CH_3

3, R = H
6, R = CH_3

Scheme 1.

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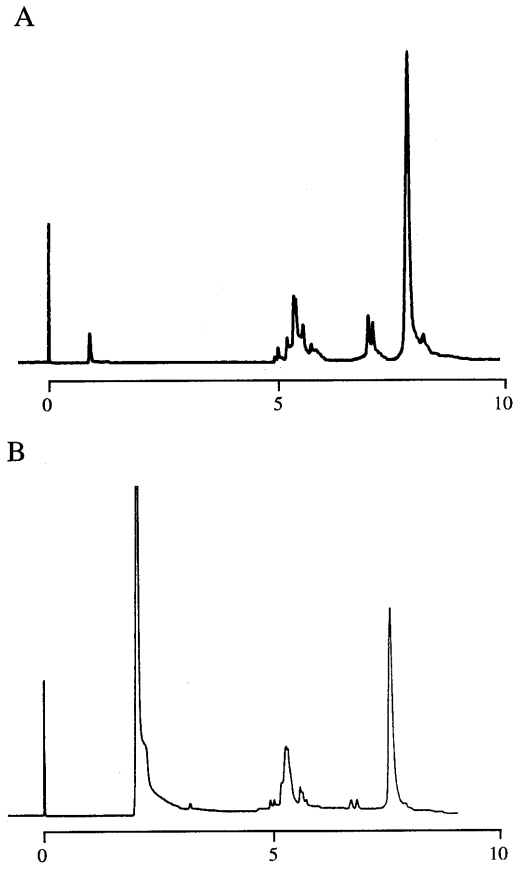

Figure 1. RP-HPLC of crude mixtures of **8** (**A**) and **7** (**B**) after deprotection and separation from supports. The large early peak in (B) is from dimethdithiocarbamate that, at that time, was added to the TEAA solution, but is better used instead in the THF wash (see Materials and Methods). A C3 column was used with a gradient of 2–80% acetonitrile:0.1M TEAA in 5 min at a flow rate of 2 ml/min.

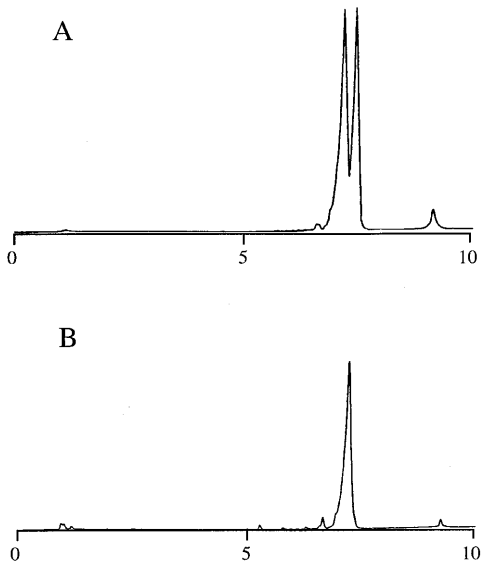

Figure 2. Anion exchange HPLC of: (**A**) coinjection of **7** and **8**; and (**B**) coinjection of **7**, after removal of its 3′-phosphate, and **8**. A Dionex NucleoPac PA-100 column (4 × 250 mm) was used with a gradient of 0–20% buffer B in buffer A in 16 min at a flow rate of 1.5 ml/min. Buffer A is 0.01 M sodium acetate and 0.01 M lithium perchlorate with 10% acetonitrile in water and buffer B is 0.01 M sodium acetate and 0.6 M lithium perchlorate with 10% acetonitrile in water.

that further optimization will yield yet milder conditions requiring fewer equivalents of Pd(0). Importantly, while no longer covalently attached to the support, the RNA fragment is not soluble in this THF/pyridine mixture, so that the reagents used in the cleavage step can be removed simply by washing the now support-adsorbed, rather than support-bound, RNA fragment. The product is then dissolved and eluted from the support with 0.1 M triethylammonium acetate (TEAA) buffer. This solution can be applied directly to a reversed-phase (RP) HPLC column for purification. Small (mg) amounts of dimethyldithiocarbamate in the TEAA help to remove any remaining Pd (16). This procedure gives the oligomer with a 3′-phosphate group which, if desired, can be removed by treatment with a phosphatase. We have used calf intestinal phosphatase (CIP) to cleave the 3′-phosphate, but for most purposes this is not necessary.

To illustrate the use of this linker in RNA synthesis, we first compared results for two 30 µmol scale preparations of an RNA decamer, one synthesized using this allyl linker (**7**) and the other synthesized using the traditional succinate linker (**8**). Figure 1 shows analytical RP-HPLC of both crude product mixtures after removal of all protecting groups except the 5′-DMT and cleavage from the supports. Each was then purified by RP-HPLC using aqueous triethylammonium acetate/acetonitrile, detritylated with 0.1 M acetic acid, purified again by RP-HPLC, desalted by RP-HPLC using aqueous ammonium bicarbonate (ABC)/acetonitrile, and converted to the sodium form using a sodium ion-exchange column. Each was found to be homogeneous by RP-HPLC and anion-exchange HPLC (data not shown). The overall yields of **7** (6.8%) and **8** (9.7%) were similar, despite the inadvertent handling loss of a portion of **7** during the purification. The difference in retention of **7** and **8** on anion exchange HPLC due to the 3′-phosphate on **7** is evident in the co-injection shown in Figure 2A. Treatment of **7** with calf intestinal phosphatase (CIP) for 1 h completely removed the phosphate group as shown by a co-injection with **8** in Figure 2B. Enzymatic degradation of both **7** and **8** using nuclease P1 and CIP gave an identical mixture of nucleosides in the expected ratio (data not shown). ICP-MS analysis of **7** showed only traces of residual Pd (2×10^{-3} atoms of Pd per RNA molecule). This is nearly identical to the level reported by Hayakawa and Noyori for use of allyl protecting groups in the synthesis of a DNA fragment (3×10^{-3} atoms of Pd per 32mer DNA molecule) (17), and confirms that Pd contamination should not be a problem for either RNA or DNA synthesis.

We then synthesized eight additional RNA molecules at 30 µmol scale (Table 1) with only minor improvements in the procedures. Overall yields of pure products ranged from 10 to 13% for octamers, and were somewhat lower for the longer molecules (up to a 13mer). As before, all RNA molecules were homogeneous by both RP-HPLC and anion exchange HPLC, and gave the correct ratio of nucleosides upon enzymatic degradation (data not shown).

The results reported here demonstrate that 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid (**3**) has significant advantages for solid-phase RNA synthesis. Additional likely applications would include DNA synthesis, the synthesis of modified nucleic acids (particularly ones that are base-sensitive), affinity columns and peptide synthesis. Because it is cleaved under mild conditions orthogonal to those used during many common reactions, the range of post-synthetic manipulations that can be carried out without cleavage from the support is extended significantly.

Table 1. RNA oligomers synthesized and overall yields of pure material

Oligomer	Yield (%)
CUGGGAGUCCp (**7**)	7
CUGGGAGUCC (**8**)	10
GGCGAGCCp	13
GAAUAGUCp	12
GAAGCGUCp	12
GAUGCGUCp	11
GGCGUGCCp	10
GCGGACGCp	7
GGACUUCGGUCCp	9
GGCGGAAUACCGCp	5

MATERIALS AND METHODS

Although the multistep method for synthesis of 9-*O*-(4,4′-dimethoxytrityl)-10-undecenoic acid (**3**) permits characterization of intermediates, the one-flask procedure is more convenient.

Multistep method

To a solution of 12 g (65 mmol) of 10-undecenoic acid, **1**, in 100 ml of dry acetone was added 7.4 ml (78 mmol) of $(CH_3)_2SO_4$ and 13.2 g (96 mmol) of K_2CO_3. The mixture was stirred at reflux for 24 h, cooled to room temperature, and filtered, and the filtrate was concentrated. The liquid residue was distilled under reduced pressure (b.p. 89°C at 1.5 mm Hg) to give 9.6 g (48 mmol, 74%) of **4**. ^{1}H NMR ($CDCl_3$) δ 5.74 (m, 1H, H_{10}), 4.90 (m, 2H, $H_{11,11'}$), 3.61 (s, 3H, OCH_3), 2.27 (t, 2H, $H_{2,2'}$), 2.00 (m, 2H, $H_{9,9'}$), 1.27–1.62 (m, 12H, CH_2's$_{3–8}$). A mixture of 1.1 g of SeO_2 (10 mmol), 50 ml of CH_2Cl_2 and 8.5 ml of 5.0–6.0 M *tert*-butylhydroperoxide in decane was stirred at room temperature for 30 min. A solution of 4.1 g (20 mmol) of **4** in 10 ml CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 2 days, washed with brine and concentrated. The liquid residue was distilled (135°C, 1.5 mm Hg) to give 1.0 g (4.6 mmol, 23%) of **5**. ^{1}H NMR ($CDCl_3$) δ 5.94 (m, 1H, H_{10}), 5.24 (m, 2H, $H_{11,11'}$), 4.12 (m, 1H, H_9), 3.66 (s, 3H, OCH_3), 2.34 (t, 2H, $H_{2,2'}$), 1.64–1.17 (m, 12H, CH_2's$_{3–8}$). To a solution of 1.5 g (7 mmol) of **5** in 25 ml of pyridine was added 3.6 g (10.7 mmol) of 4,4′-dimethoxytrityl chloride (DMTCl). The reaction mixture was stirred at room temperature overnight and filtered. The filtrate was concentrated, and the residue purified by chromatography on silica gel using a gradient of 2% pyridine in petroleum ether to 2% pyridine, 2% methanol and 20% ethyl acetate in petroleum ether to give 3.46 g (6.7 mmol, 96%) of **6** as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ 7.51–6.77 (m, 13H, ArH's), 5.72 (m, 1H, H_{10}), 4.83 (m, 2H, $H_{11,11'}$), 3.90–3.79 (m and s, 7H, H_9 and Ar-OCH_3's), 3.66 (s, 3H, OCH_3), 2.31 (t, 2H, $H_{2,2'}$), 1.65–1.09 (m, 12H, CH_2's$_{3–8}$). To a mixture of 25 ml of THF and 5 ml of water was added 3.46 g (6.7 mmol) of **6** and 1.1g (20 mmol) of KOH. The reaction mixture was refluxed overnight, cooled and partitioned in a mixture of 150 ml each of water and ethyl ether. The aqueous layer was then extracted with a 150 ml portion of a 1:1 mixture of ethyl ether and ethyl acetate. The latter organic layer was concentrated to give 3.2 g (5.9 mmol, 88%) of the potassium salt of **3** as a wax. ^{1}H NMR ($CDCl_3$), δ 7.51–6.75 (m, 13H, ArH's), 5.71 (m, 1H, H_{10}), 4.83 (m, 2H, $H_{11,11'}$), 3.90–3.79 (m and s, 7H, H_9 and Ar-OCH_3's), 2.82 (b, 2H, $H_{2,2'}$), 1.50–1.15 (m, 12H, CH_2's$_{3–8}$). IR (film) cm^{-1}, 3452, 2930, 2856, 1739, 1437, 1363, 1247, 1199, 1173, 992, 920.

One-flask method

To a mixture of 0.36 g (3.2 mmol) of SeO_2 and 50 ml of methylene chloride was added 6.5 ml of 5–6 M *tert*-butyl hydroperoxide in decane. The mixture was stirred at room temperature for 30 min and a solution of 2.0 g (11 mmol) of 10-undecenoic acid (**1**) in 10 mL of CH_2Cl_2 was added dropwise. The mixture was stirred at room temperature for 2 days, washed successively with water and brine, dried over Na_2SO_4, and concentrated to an oil (oil pump, 50°C). GC/MS showed that this material contained ~25% of unreacted **1** but was used in the next step without purification. To this crude **2** dissolved in 25 ml of pyridine was added 4.5 g (13 mmol) of 4,4′-dimethoxytrityl chloride. After 16 h at room temperature the mixture was filtered, concentrated and the product purified by chromatography on silica gel using a gradient of 2% pyridine in petroleum ether to 2% pyridine, 2% methanol and 30% ethyl acetate in petroleum ether. Concentration of the product-containing fractions gave 2.4 g (4.1 mmol, 38% from **1**) of **3** as the pyridinium salt**.** MS (+FAB) m/e, 502.3 (M^+, $C_{32}H_{38}O_5$ requires 502.7), (–FAB) m/e 501.5 ($M\text{-}H^+$, $C_{32}H_{37}O_5$ requires 501.7).

Attachment of 3 to amino-functionalized polystyrene/polyethylene glycol

To 3 ml of CH_2Cl_2 was added 220 mg (0.38 mmol) of **3**, 130 mg (0.63 mmol) of dicyclohexylcarbodiimide and 600 mg of polystyrene/polyethylene glycol (PS/PEG) (13). The viscous mixture was shaken at room temperature for 16 h, filtered, and the support washed successively with CH_2Cl_2, methanol, CH_2Cl_2 and diethyl ether. The unreacted amino groups remaining on the support were capped by shaking with a pyridine solution containing 10% acetic anhydride and 1% *N*-methyl imidazole for 2 h at room temperature. The mixture was filtered, and the support was washed successively with CH_2Cl_2, methanol, CH_2Cl_2 and diethyl ether and then dried.

Determination of loading of 3 on PS/PEG

To a 100–120 mg sample of the derivatized support was added 2–3 ml of 2.5% dichloroacetic acid (DCA). After 2 min the mixture was filtered and the support washed with three additional portions of DCA. The combined filtrates were diluted to 500 ml and the absorbance measured at 502 nm. The loading was calculated using an extinction coefficient of 9×10^4 l cm^{-1}mol^{-1} and typically ranged from 150–180 μmol/g.

Deprotection of 7

To the support was added 4 ml of a mixture of concentrated aqueous ammonia and 95% ethanol (3:1). The mixture was sealed and maintained at room temperature for 16 h. The solution was removed and the support washed three times with 10 ml of THF. The support was then deprotected with 4 ml of 1.0 M TBAF. After 4 h the TBAF solution was removed, and a fresh 4 ml portion of TBAF was added. After 16 h the TBAF was removed and the support washed three times with 10 ml THF.

Improved deprotection of other RNA molecules using the allyl linker

To the support was added 4 ml of a mixture of concentrated aqueous ammonia and 95% ethanol (3:1). The mixture was sealed and shaken at 58°C in an oven (Boekel, incubator and shaker) for 6 h. The solution was removed and the support washed three times

with 5 ml of DMF and three times with 5 ml of THF. Desilylation was achieved by either of two procedures. (i) The support was treated with 5 ml TEA/3HF (7) at 58°C with shaking for 4 h. The solution was removed and the support washed three times with 5 ml DMF and three times with 5 ml THF. (ii) The support was treated with 3 ml of *N*-methylpyrrolidinone, 1.5 ml TEA, and 2.0 ml TEA/3HF (6:3:4) (18) at 58°C with shaking for 3 h. The solution was removed and the support washed three times with 5 ml DMF and three times with 5 ml THF.

Cleavage of 7 from the support

To the support-bound **7** (30 µmol scale) was added 35 mg (0.03 mmol) of tetrakis(triphenylphosphine) palladium (Aldrich 21,666-6), 31 mg (0.12 mmol) triphenylphosphine, and a 4 ml portion of *n*-butylammonium formate in pyridine and THF. This solution was prepared by adding 160 mg (3.5 mmol) of formic acid to 220 mg (3.5 mmol) of *n*-butylamine in 5 ml THF, followed by addition of formic acid to produce a clear solution, and then addition of 1 ml pyridine. The heterogeneous reaction mixture was sparged with nitrogen, sealed and heated at 60°C for 4 h. After being cooled to room temperature, the mixture was centrifuged, the supernatant removed, and the solid washed twice with THF. The product was then eluted from the support with three 5 ml portions of 0.1 M TEAA:95% ethanol (2:1) containing 1–3 mg of dimethyldithiocarbamate. RP-HPLC of the crude mixture is shown in Figure 1B.

Improved cleavage of other RNA molecules from the support

The other molecules were cleaved from the support as described above except that (i) as few as 0.5 equiv. of tetrakis(triphenylphosphine) palladium was used, (ii) the THF wash contained 0.001g/ml dimethyldithiocarbamate sodium salt, and (iii) no dimethyldithiocarbamate was added to the TEAA/ethanol mixture.

Deprotection of 8 with cleavage from the PS/PEG support

The support was treated with concentrated aqueous ammonia and 95% ethanol (3:1) at room temperature for 16 h in a 50 ml centrifuge tube. The mixture was centrifuged, the supernatant removed, and the solid washed three times with water. The combined solutions were concentrated to remove most of the ammonia and lyophilized. The residue was treated with 5 ml 1.0 M TBAF in THF for 12 h, and the mixture was concentrated to a gum. RP-HPLC of the crude mixture is shown in Figure 1A.

Purification of oligomers

A trityl-on purification of each oligomer was carried out on a Waters C18 reversed-phase column (25 × 100 mm RCM cartridge) using a gradient of 2–40% acetonitrile: 0.1 M TEAA in 45 min at a flow rate of 4 ml/min. The appropriate fractions were combined, diluted with an equal volume of water and lyophilized. The residue was dissolved in water and an equal volume of 0.2 M acetic acid was added to give a solution with a pH of ~3.2. The detritylation was complete after 20 min (HPLC). The pH was adjusted to 6 and the solution was lyophilized. The residue was dissolved in 0.1 M TEAA, filtered and purified using a Beckman Ultrapore C3 column (10 mm × 250 mm) using a gradient of 2–20% acetonitrile:0.1 M TEAA in 45 min at a flow rate of 2 ml/min. The appropriate fractions were combined, diluted with an equal volume of water and lyophilized. The residue was dissolved in 0.1 M ammonium bicarbonate (ABC) and chromatographed on the C18 column using a gradient of 2–40% acetonitrile: 0.1 M ABC in 35 min at a flow rate of 4 ml/min. The appropriate fractions were combined, diluted with an equal volume of water and lyophilized. The residue was dissolved in water and passed through a Bio-Rad AG50W-X4 sodium-form ion-exchange column. The appropriate fractions were combined and lyophilized to give the sodium salt.

Enzymatic conversion of 7 to 8

To a 1.0 OD_{260} sample of **7** in 1 ml 0.1 M TEAA was added 0.04 U calf intestinal phosphatase (CIP, Sigma P-5778). Ion-exchange HPLC showed that the 3′-phosphate group was completely removed within 1 h, and that there was no further change after an additional 7 h. Figure 2 shows anion-exchange HPLC coinjections of **7** and **8** before (A) and after (B) removal of the 3′ phosphate of **7**.

Enzymatic degradation of 7 and 8

To a 1 OD_{260} sample of **7** or **8** in 1 ml 0.1 M TEAA was added 1 U nuclease P1. After 1 h at room temperature, the pH was adjusted to 9–10 using 10% sodium carbonate, and 1 U CIP was added. Degradation to ribonucleosides was complete within 1 h.

ICP-MS

A Fisons VG PlasmaQuad PQ2 Turbo Plus ICP-MS was used to detect the presence of trace amounts of Pd(0) in RNA made with the allyl linker.

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