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Fast and simple purification of chemically modified hammerhead ribozymes using a lipophilic capture tag

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ABSTRACT

A new type of 5′-lipophilic capture tag is described, enabling the facile reverse phase HPLC purification of chemically modified hammerhead ribozymes (oligozymes) whilst still carrying the 2′-*O*-*tert*.-butyldimethylsilyl protection of the essential riboses. In its most convenient form, the capture tag consists of a simple diol, such as hexan-1,6-diol, which at one end is attached via a silyl residue to a highly lipophilic entity such as tocopherol (vitamin E) or cholesterol, and the other end is functionalized as a phosphoramidite. This lipophilic capture tag is added as the last residue in the solid-phase synthesis of chemically modified hammerhead ribozymes. Cleavage from the support and release of all protecting groups except for the silyl groups is achieved with ethanolamine/ethanol. The crude product is then loaded directly on to a reverse phase HPLC column. Separation of failure peaks from full length product is achieved easily using a short run time. The retarded product peak is collected, lyophilized, desilylated in the normal way and then desalted. This method removes the lipophilic capture tag yet leaves behind the hexanediol entity which helps protect the compound against degradation by 5′-exonucleases. The purity of the product as judged by analytical anion-exchange HPLC and capillary gel electrophoresis is generally better than 95% full-length, and yields of 2–4 mg from a 1 μmol scale synthesis are routine. In addition, the method can be readily scaled up, an important feature for the development of such chemically modified ribozymes as potential therapeutics.

The hammerhead ribozyme self-cleaving motif was originally identified in plant pathogens such as the avocado sunblotch viroid (1). Subsequently, it was shown that dissection of the hammerhead motif into a catalytic and a substrate part allows the specific cleavage of RNA sequences (2,3). Since then, application of *trans*-cleaving hammerhead ribozymes for gene inactivation by specific cleavage of mRNAs has become a major topic of interest (4,5). We and others are interested in the use of chemically modified hammerhead ribozymes for this purpose. In general, these molecules contain five residual ribonucleotides required for maintaining catalytic activity. These are positions G5, A6, G8, G12 and A15.1, based on the now standard numbering scheme (6). Recently, we extended the cleavage rules for the hammerhead ribozyme from $N^{16.2}U^{16.1}H^{17}$ to $N^{16.2}C^{16.1}H^{17}$ by using inosine instead of adenosine at position 15.1 (7), and are interested in applying chemically modified versions of these new ribozymes to a variety of biological problems in both cell and animal model systems.

The modified ribozymes are generated by standard methods on solid-phase; however, a major synthetic hurdle is the separation of failure sequences from the desired oligonucleotide. Current purification methods available include anion-exchange HPLC, polyacrylamide gel electrophoresis (PAGE), reverse phase HPLC, ion-pair chromatography and affinity chromatography. Each of these methods has one or more shortcomings, which will be exemplified below.

Purification of oligoribonucleotides and/or chemically modified ribozymes using anion-exchange chromatography with Pharmacia Mono Q or Dionex NucleoPac columns and eluting with a salt gradient such as sodium or lithium perchlorate has been described (8,9). However, single base resolution of chemically modified hammerhead ribozymes, which are generally in the range of 32–38 nt in length, is typically not possible. In addition, problems of secondary structure necessitate separating at elevated temperature or require the use of an organic cosolvent. The run times are long if good resolution is required, the columns have a low loading capacity and moreover are extremely expensive. Thus current anion-exchange HPLC methods are problematic for purifying quantities in excess of 1–2 mg.

Purification of chemically synthesized oligoribonucleotides and 2′-*O*-methyl modified hammerhead ribozymes has been achieved by using the traditional trityl-on reverse phase HPLC method followed by a second purification step by ion-exchange HPLC (9). However, this method is very time consuming and, due to the intrinsic instability of the trityl group, losses occur due to premature detritylation. Furthermore, in any purification strategy the potential for product loss is increased with each successive manipulation. Since the *tert*.-butyldimethylsilyl protecting groups on the essential riboses impart substantial lipophilicity alone, they must be removed from the ribozyme in order to achieve an efficient trityl-on purification by reverse phase HPLC (9). An additional problem with this method is that the fluoride reagent must be removed from the material to be purified if silica based reverse phase HPLC columns are to be used. Following purification, the trityl group has to be removed under acidic

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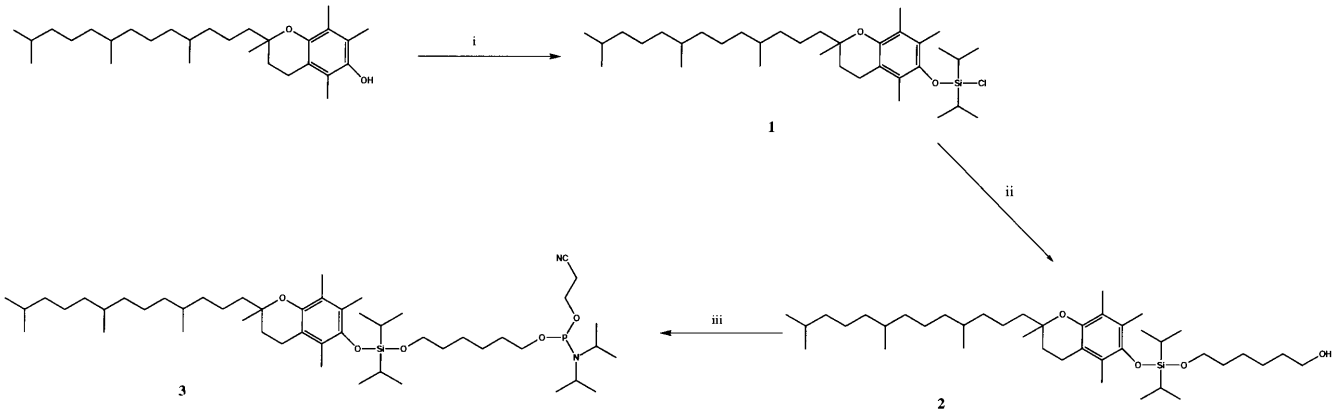

Figure 1. Reaction scheme for the preparation of the lipophilic purification handle. Reagents and conditions: (i) diisopropyldichlorosilane and triethylamine in dichloromethane, 48 h at room temperature; (ii) 1,6-hexanediol, triethylamine and 4-dimethylaminopyridine in dichloromethane/DMF, overnight at room temperature; (iii) 2-cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine and *N*,*N*-diisopropylethylamine in dichloromethane, 0°C for 15 min then room temperature, 45 min.

conditions, and care must be taken to avoid possible acid catalyzed migration of 3′,5′-internucleotide linkages at ribose positions as well as strand cleavage (10). For these reasons, the above method is not satisfactory for obtaining high yields of very pure modified ribozymes in a reasonable time.

Reverse phase ion-pair chromatography of unprotected chimeric hammerhead ribozymes on polystyrene based columns has been used as a purification method (11) and although the columns have a reasonable capacity, resolution is poor and run times are long.

PAGE can be used for small scale purifications of chemically modified ribozymes. A method for larger scale purification which combines gel electrophoresis with column chromatography has been described (12) and was used to purify 6.5 mg of a 34 residue long transcribed ribozyme in a single run. This method is limited because the run time is ~20 h and it is still restricted to relatively small amounts of material.

One could also imagine the use of affinity purification using sequences complementary to the 5′- and/or 3′-ends of the ribozyme. Such a method has been used for the batchwise purification of specific tRNAs (13). The major disadvantage here is that a different affinity matrix would be required for every ribozyme synthesized. Other affinity based methods utilizing hapten and antibody based systems such as biotin-avidin, although adequate for small scale, are impractical for large scale work due to the prohibitive cost involved.

Herein we describe a cheap new purification method that is fast, reliable and can be easily scaled up. Since only low pressure equipment is required, scale up to kilogram quantities can be expected without problems.

5′-*O*-Dimethoxytrityl-2′-*O*-allylribonucleoside-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidites), 5′-*O*-dimethoxytrityl-2′-*O*-methylribonucleoside-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidites) and 5′-*O*-dimethoxytrityl-2′-*O*-*tert*.-butyldimethylsilylribonucleoside-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidites) bearing *tert*.-butylphenoxyacetyl protection of the exocyclic amino functions of adenine, cytosine and guanine were obtained from PerSeptive Biosystems; 5′-*O*-dimethoxytrityl-2′-*O*-*tert*.-butyldimethylsilylinosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) bearing an N^1-pivaloyloxymethyl protecting group was also obtained from PerSeptive Biosystems. All other synthesis chemicals were from Perkin Elmer/Applied Biosystems or PerSeptive Biosystems, Hamburg. Molar extinction coefficients for oligonucleotides were calculated according to the nearest neighbour model (14).

^{13}C and ^{31}P NMR spectra were recorded on a Bruker AC 200 spectrometer using tetramethylsilane and external trimethyl phosphate as references. Chemical shifts are reported in parts per million (p.p.m.) and are positive when downfield of the standard. The various intermediates were synthesized as follows and the synthetic route used for the preparation of the lipophilic purification tag monomer is illustrated in Figure 1.

Diisopropyl(DL-α-tocopheryloxy)chlorosilane (1)

A solution of DL-α-tocopherol (21.5 g, 50 mmol) in dry dichloromethane (100 ml) was added dropwise with stirring during 30 min to a solution of diisopropyldichlorosilane (9.05 ml, 50 mmol) and triethylamine (7.65 ml, 55 mmol) in dichloromethane (500 ml) at 0°C under anhydrous conditions. The reaction was allowed to warm up to room temperature and stirred for 48 h. The solvent was evaporated *in vacuo* and petroleum ether/diethyl ether (200 ml, 1:1 v/v) was added to the residue and stirred until the oil dissolved and the triethylamine hydrochloride precipitated. The salt was filtered off, washed with petroleum ether/diethyl ether and the combined filtrate and washings were evaporated *in vacuo* to an oil, which was dissolved in anhydrous dichloromethane (50 ml) and used for the next step without purification, assuming a quantitative reaction.

1-[Diisopropyl(DL-α-tocopheryloxy)silyl]oxy-6-hydroxyhexane (2)

A solution of compound **1** (25 mmol) in dichloromethane (25 ml) was added to a solution of 1,6-hexanediol (14.77 g, 125 mmol), triethylamine (5.2 ml, 37.5 mmol) and 4-dimethylaminopyridine (460 mg, 3.75 mmol) in dichloromethane/*N*,*N*-dimethylformamide (100 ml, 3:1 v/v) and the reaction mixture was stirred overnight at room temperature with exclusion of moisture. The solvent was removed *in vacuo* and the residue dissolved in dichloromethane (50 ml), washed with aqueous sodium bicarbonate solution, dried

(sodium sulphate), filtered and evaporated to dryness *in vacuo*. Purification on a column of silica gel (5 × 13 cm) eluted with petroleum ether/ethyl acetate (4:1 v/v) afforded compound **2** (7.3 g, 44%) as a colourless oil of R_f 0.83 on silica gel TLC in petrol/ethyl acetate (2:1 v/v). ^{13}C NMR spectrum ($CDCl_3$) δ: 145.82, 144.91, 125.16, 122.76, 122.30, 117.11 and 74.28 (*tert*. Cs tocopheryl), 39.52, 39.32, 37.35 (4Cs), 31.73, 24.74, 24.36 and 20.91 (2Cs, CH_2s of tocopheryl), 32.66 (2C) and 27.88 (CHs, tocopheryl), 23.62, 22.60 (2Cs), 19.60 (2C), 13.72, 12.84 and 11.86 (CH_3s of tocopheryl), 63.18 (C-1), 62.56 (C-6), 32.54 (C-2 and C-5), 25.53 (C-3 and C-4), 17.26 (isopropyl CH_3s) and 12.84 p.p.m. (isopropyl CHs).

1-[Diisopropyl(DL-α-tocopheryloxy)silyl]oxy-6-(2-cyanoethoxy *N*,*N*-diisopropylaminophosphinoxy)hexane (3)

Compound **2** (7.3 g, 11 mmol) was dried by several evaporations of anhydrous toluene *in vacuo*, then dissolved in dichloromethane (100 ml) under argon and *N*,*N*-diisopropylethylamine (5.7 ml, 33 mmol) was added and the solution was cooled in an ice-bath. 2-Cyanoethoxy *N*,*N*-diisopropylaminochlorophosphine (2.91 ml, 13.2 mmol) was added slowly via a syringe and the reaction was stirred at 0°C under anhydrous conditions. After 45 min TLC on silica gel plates pre-run in petroleum ether/ethyl acetate (2:1 v/v) containing 2% triethylamine to avoid decomposition of the product showed complete reaction with a new spot of R_f 0.90. The reaction was diluted with dichloromethane, washed with aqueous sodium bicarbonate solution, dried (sodium sulphate), filtered and evaporated to dryness *in vacuo*. Purification on a Florisil column (5 × 13 cm) eluting with petrol/ethyl acetate (4:1 v/v) containing 1% triethylamine afforded compound **3** (7.7 g, 81%) as a colourless oil. ^{31}P NMR spectrum ($CDCl_3$) δ: 146.20 p.p.m.

2′-*O*-Allyl and 2′-*O*-methyl modified ribozymes containing five residual purine ribonucleotides (that is G5, A6, G8, G12 and A or I15.1) were synthesized on a 1 µmol scale by solid-phase β-cyanoethyl phosphoramidite chemistry (15) using the 2′-*O*-*tert*.-butyldimethylsilyl (TBDMS) protection strategy for the ribonucleotides (16,17). Syntheses were performed on aminomethylpolystyrene bearing an inverted thymidine linkage (18) using a standard RNA cycle except for a cap-oxidize-cap procedure following each monomer addition. After adding the last desired 2′-*O*-alkyl-ribonucleotide at the 5′-end of the oligomer, a further synthesis cycle was performed using monomer **3** as a 0.1 M solution in acetonitrile/dichloromethane (1:1 v/v) and coupling for 15 min using the standard 1*H*-tetrazole solution. Following the cap-ox-cap part of the cycle, the column was washed thoroughly with acetonitrile and blown dry.

The oligomer was cleaved from the support and all base labile protecting groups (2-cyanoethyl and 4-*tert*.-butylphenoxyacetyl) were conveniently removed by treatment with anhydrous ethanolamine/ethanol (0.5 ml, 1:1 v/v) for 2 h at 60°C or 4 h for sequences containing inosine (19,20). This procedure leaves the silyl protecting groups intact. The deblock solution containing partially protected ribozyme was then removed and the synthesis cartridge was washed twice with 0.5 ml of ethanol/acetonitrile/water/DMSO (3:3:2:2 by vol). The solution was then injected directly into a reverse phase HPLC column (Pharmacia Source 15 RPC 10/10 or Hamilton PRP-1 305 × 7 mm) equilibrated at 15% B buffer (buffer A is 10% acetonitrile in 0.1 M aqueous triethylammonium acetate, pH 7.5; buffer B is 95% acetonitrile in 0.1 M aqueous triethylammonium acetate pH 7.5). The elution conditions were 15% B for 1.5 CV (column volumes), 15–35% B for 0.5 CV, 35% B for 1.5 CV, 35–100% B for 3 CV, 100% B for 0.5 CV, 100–15% B for 0.5 CV and 15% B for 1 CV using flow rates of 7 and 4 ml/min for the Source and PRP-1 columns, respectively. Reverse phase HPLC purification either on a Hamilton PRP-1 column or on a self-packed Pharmacia Source column is both fast and very easy as is exemplified by the HPLC traces shown in Figures 2a and 3a, which illustrate examples of a 2′-*O*-methyl modified ribozyme containing five residual ribonucleotides and a 2′-*O*-allyl modified ribozyme containing six residual ribonucleotides, respectively. The failure peak eluted after ~1 min and the well separated product peak eluted after ~7 min. In general, the yield of full-length product is ~50–60%. The desired product peak eluting at ~80% B was collected and lyophilized in a Speedvac concentrator. The dried down still silyl protected ribozyme was then dissolved in triethylamine tri(hydrofluoride)/DMSO (0.4 ml, 3:1 v/v) in a sterile Eppendorf tube and kept at 60°C for 2 h (21). When cool, the fully deprotected ribozyme was precipitated by addition of 1-butanol (1.5 ml), kept at room temperature for 2 h and the precipitate was recovered by centrifugation at 6000 r.p.m. for 10 min. The pellet was washed with pure acetone (2 × 1 ml), spun down for 5 min and then dried in a SpeedVac. The dried ribozyme was then dissolved in 1 M sodium acetate solution (0.2 ml, pH 8) to which was added 5 M aqueous sodium perchlorate (0.1 ml). Ice-cold acetone (1.6 ml) was then added and the mixture was kept at –20°C for 30 min. The precipitate of sodium salt of the modified ribozyme was collected by centrifugation at V_{max} on a table top centrifuge. The resulting pellet was washed with pure acetone (2 × 0.5 ml) to remove residual sodium perchlorate, and as a final precaution was desalted on a NAP-10 column using sterile water as eluant. The desalted ribozyme was then lyophilized and stored at –20°C.

Analytical anion-exchange HPLC of purified chemically modified ribozymes was performed on a Dionex Nucleopac PA-100 column (4 × 250 mm) at 55°C eluting at 2 ml/min with a gradient of sodium perchlorate in 25 mM Tris–HCl buffer ph 7.0 in water/acetonitrile (9:1 v/v) containing 1 mM EDTA. Buffer A contained 10 mM sodium perchlorate and buffer B contained 500 mM sodium perchlorate. The results are illustrated in Figures 2b and 3b. The following gradient conditions were used: 8% B for 2 min; 8–23% B during 0.5 min; 23% B for 1 min; 23–65% B during 6 min; 65–100% B during 0.25 min; 100% B for 1.25 min; 100–8% B during 0.25 min and then 8% B for 2 min to re-equilibrate the column.

The homogeneity of the chemically modified hammerhead ribozymes was analyzed by capillary gel electrophoresis on a Biofocus 3000 Capillary Electrophoresis system (Bio-Rad), (Figs 2c and 3c) Probes were analyzed under denaturating conditions using Dynamic Sieving Buffer (Bio-Rad 148–502) and 8 M urea and coated capillaries (44 cm × 15 µm), using an electrokinetic injection at 5 kV for 8 s, and peaks were detected by UV absorption at 260 nm. In addition, all modified ribozymes used were characterized by mass spectroscopy on a Voyager DE Biospectrometry workstation (PerSeptive Biosystems). For MALDI TOF MS analysis, 0.04 A_{260} U in 2 µl water was microdialyzed using a 0.025 µm membrane. An aliquot of 1 µl from the resulting dialyzed sample was mixed with 2,4,6-trihydroxyacetophenone: ammonium citrate matrix and samples were prepared according to the protocol provided by PerSeptive. Between 150 and 256 shots were averaged in the positive ion

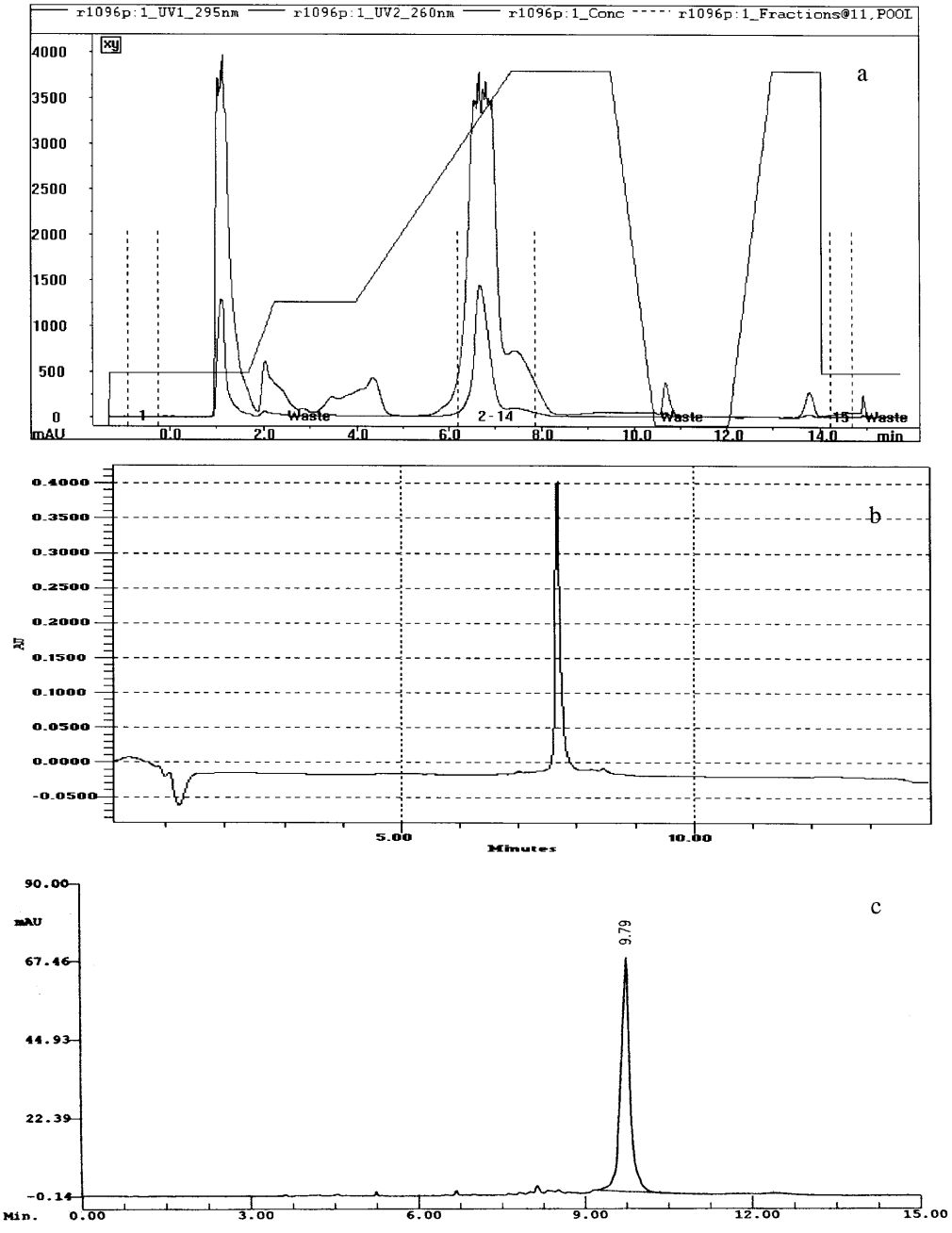

Figure 2. HPLC and CGE traces illustrating the purification and subsequent analysis of 5′-hex-GCGACCCUgaUgAGGCCGUGAGGCCgAAiCAUUC(iT), whereby A, C, G and U are 2′-*O*-methylribonucleotides; a, g and i are ribonucleotides; hex is the terminal hexanediol residue and iT is an inverted thymidine residue. (**a**) Reverse phase HPLC trace of 'silyl-on' purification, i.e. containing the lipophilic capture tag and the 2′-*O*-TBDMS groups, using a Pharmacia Source 15 RPC 10/10 column and eluting with a gradient of acetonitrile in aqueous 0.1 M triethylammonium acetate buffer pH 7.5 at a flow rate of 7 ml/min. (**b**) Analytical anion-exchange HPLC trace of purified fully deprotected oligomer run on a Dionex Nucleopac PA-100 column (4 × 250 mm) at 55°C eluting at 2 ml/min with a gradient of sodium perchlorate in 25 mM Tris–HCl buffer pH 7.0 in water/acetonitrile (9:1 v/v) containing 1 mM EDTA. (**c**) Dynamic sieving capillary electropherogram of purified fully deprotected oligomer.

mode for each spectrum using an acceleration voltage of 256 kV. Spectra were calibrated with two external standards, $dT(pdT)_{25}$ and $dT(pdT)_{50}$. The measured molecular weights for the 2′-*O*-methyl and 2′-*O*-allyl modified ribozymes were 11 859.6 (calculated 11 842.4) and 12 572.5 (calculated 12 551.5) Da, respectively, using only external standards for calibration. The analyses show that the products are of very high purity, >95% full-length by capillary electrophoresis and analytical anion-exchange HPLC. Morever, the new method gives very high recoveries of product with isolated yields of 2–4 mg from a 1 µmol scale synthesis. When we used traditional purification methods, such as ion-exchange HPLC of fully deprotected material, the recovered yields were generally only ~1 mg and the percentage of full-length product was often not more than 80–85% due to the difficulties of removing failure peaks just a few bases shorter than the desired product.

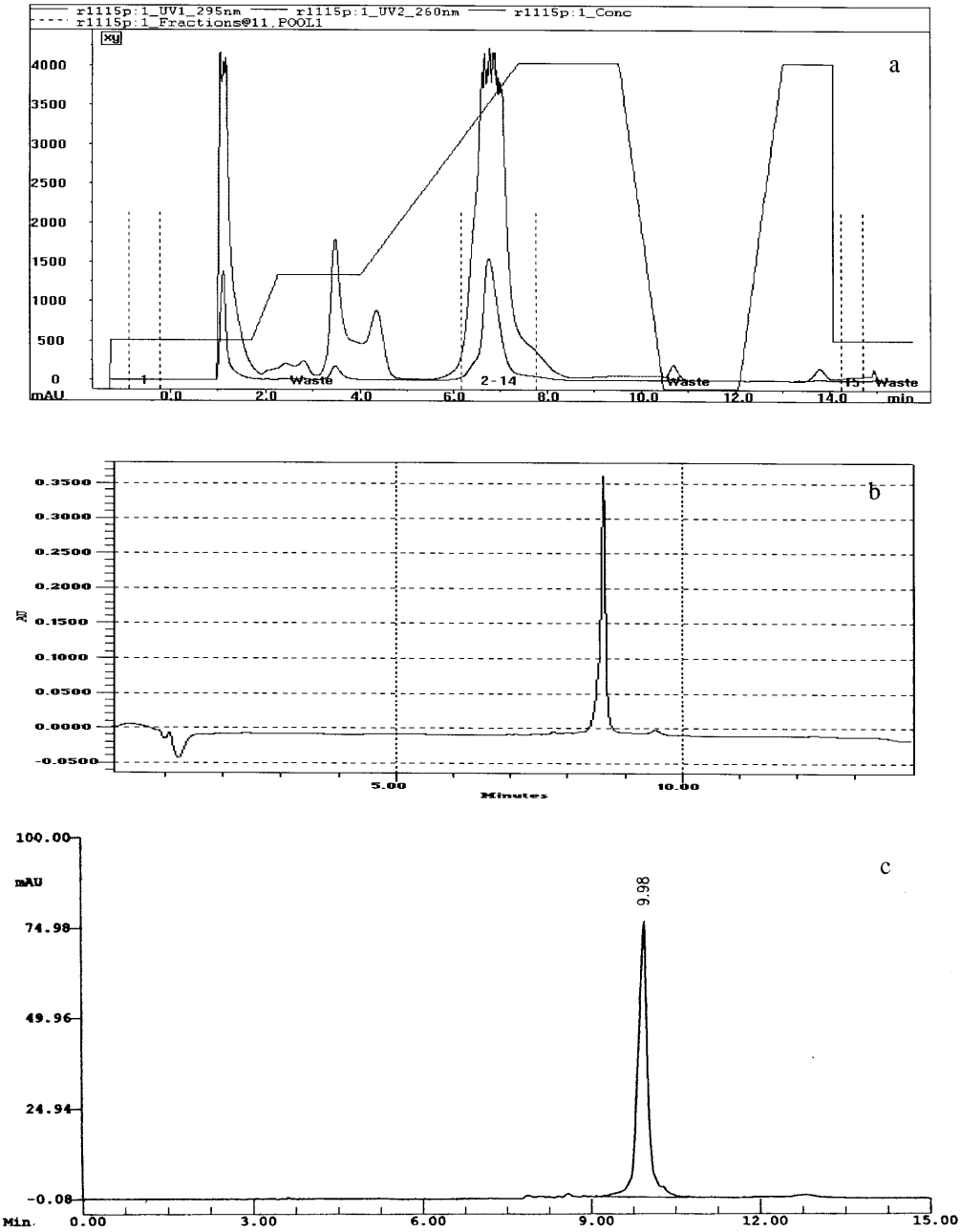

Figure 3. HPLC and CGE traces illustrating the purification and subsequent analysis of 5′-hex-GCGACCCUgaUgaGGCCGUGAGGCCgAAaCAUUC(iT), whereby A, C, G and U are 2′-*O*-allylribonucleotides; a and g are ribonucleotides; hex is the terminal hexanediol residue and iT is an inverted thymidine residue. (**a**) Reverse phase HPLC trace of 'silyl-on' purification, i.e. containing the lipophilic capture tag and the 2′-*O*-TBDMS groups, using a Pharmacia Source 15 RPC 10/10 column and eluting with a gradient of acetonitrile in aqueous 0.1 M triethylammonium acetate buffer pH 7.5 at a flow rate of 7 ml/min. (**b**) Analytical anion-exchange HPLC trace of purified fully deprotected oligomer run on a Dionex Nucleopac PA-100 column (4 × 250 mm) at 55°C eluting at 2 ml/min with a gradient of sodium perchlorate in 25 mM Tris–HCl buffer pH 7.0 in water/acetonitrile (9:1 v/v) containing 1 mM EDTA. (**c**) Dynamic sieving capillary electropherogram of purified fully deprotected oligomer.

If no residual linker is required at the 5′-end of the ribozyme then the lipophilic silyl residue can be attached directly to the 5′-hydroxyl group of a suitably protected 2′-*O*-alkylribonucleoside. Other diol linkers which have been used successfully in place of hexane-1,6-diol are diethylene glycol and benzene-1,4-dimethanol. Moreover, the use of diols such as diethanolamine allows subsequent post-synthetic labeling of the secondary amino group with a variety of commercially available reporter groups. For this purpose the building block employed is 1-[diisopropyl(DL-α-tocopheryloxy)silyl]oxy-5-(2-cyanoethoxy *N*,*N*-diisopropylamino-phosphinoxy)-3-trifluoroacetyl-3-azapentane.

Previously, vitamin E had been permanently attached to antisense oligonucleotides for improving cellular uptake, and its retardation effect on reversed phase HPLC had been noted (22). In the method described here, the use of a highly lipophilic, cleavable tag enables the rapid purification of chemically

modified hammerhead ribozymes in high yield and purity. Moreover, the presence of the linker had no measurable effect on the cleavage activity of the ribozymes (7), and their activity was not different when they were purified by alternative procedures, such as PAGE. Recovered yields of purified product are generally in the range of 2–4 mg from a 1 µmol scale synthesis, and the purity based on analytical anion-exchange HPLC and capillary gel electrophoresis is generally >95% full-length. The simplicity of the method, which has so far been applied successfully to chemically modified ribozymes containing 2′-*O*-methyl- or 2′-*O*-allyl-ribonucleotides with up to eight ribonucleotides in the catalytic core, should enable scale up to kilogram quantities, since only low pressure equipment is required. Such quantities are essential when considering the development of such compounds as potential therapeutics. During 1994, the 5 mmol scale synthesis of a 25mer oligodeoxyribonucleotide phosphorothioate was described and the product was purified trityl on by reverse phase HPLC (23). After detritylation, 12 g of product were obtained. Since then, syntheses at the 100 mmol scale and beyond have been made delivering several hundred grams of product in a single run, and syntheses at the 1 mol scale will soon become routine.

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