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Synthesis of a ^{35}S-labeled dinucleoside phosphorothioate prodrug, an orally bioavailable anti-HBV agent

Sung-Whi Rhee,[a] Radhakrishnan P. Iyer,[b] John E. Coughlin,[b] Seetharamaiyer Padmanabhan,[b] Jeremiah P. Malerich,[a] and Mary J. Tanga[a]*

The ^{35}S-labeled, dinucleoside phosphorothioate 1, an orally available agent against hepatitis B virus, was prepared in eight steps with high specific activity and radiochemical purity. Radiolabeled 3*H*-benzodithiole-3-one-1,1-dioxide was synthesized in four steps from $^{35}S_8$ and was used as the sulfurizing reagent.

Keywords: dinucleoside; phosphorothioate; hepatitis B virus; solid-supported synthesis; antiviral

Introduction

Acute and chronic liver infections caused by hepatitis viruses HBV and HCV constitute a major worldwide public health crisis affecting nearly 550 million people worldwide. There are an estimated 350 million chronic carriers of HBV worldwide of which about 1 million people die each year from chronic HBV.[1] Liver cancer patients as well as significant numbers of liver transplant recipients have a continued need for effective anti-HBV therapy. Although several anti-HBV drugs are in clinical use, significant unmet medical need exists due to dose-limiting toxicities and antiviral resistance. Safer and more effective anti-HBV drugs with novel mechanisms of action that can be used alone and in combination with other drugs are urgently needed.

Phosphorothioate dinucleotides and trinucleotides are a new class of anti-HBV compounds with novel mechanism(s) of action and potent antiviral activity *in vitro* and *in vivo*.[2,3] We recently reported that the alkyloxycarbonyl prodrug **1**, a representative of dinucleoside phosphorothioate, is an orally bioavailable anti-HBV agent.[4] In this paper, we report the synthesis of ^{35}S-labeled **1** as an [R_P,S_P]-diasteromeric mixture that was employed in absorption, distribution, metabolism, and excretion (ADME) studies in rats.[5]

Results and discussion

A key step in the synthesis of the target compound is the oxidative sulfurization of the support-bound dinucleoside phosphite triester **9**. The use of elemental $^{35}S_8$ in sulfurization reactions is cumbersome and inefficient because it involves the use of toxic reagents such as CS_2, collidine, and lutidine as the reaction medium.[6] Hence, we considered the use of radiolabeled 3*H*-benzodithiole-3-one-1,1-dioxide (3*H*-BD), a commonly used sulfurizing reagent in oligonucleotide synthesis.[7]

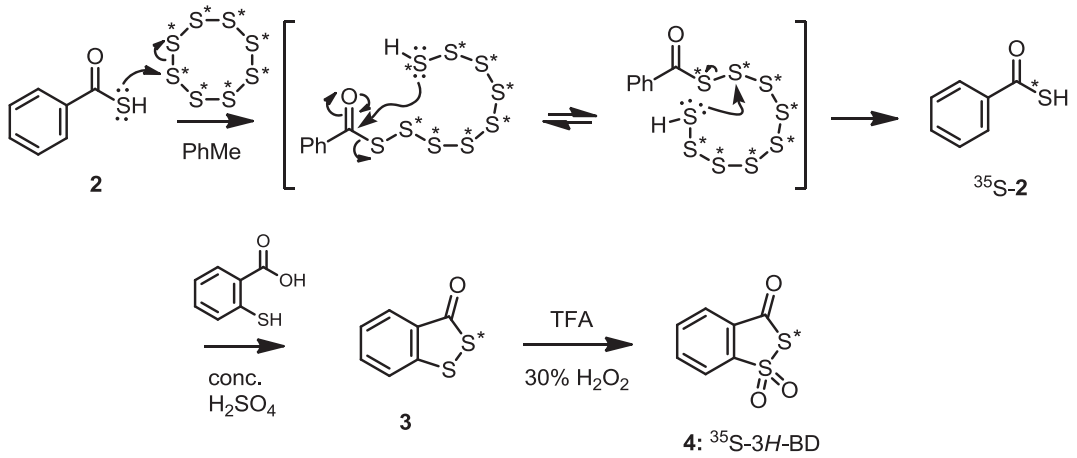
^{35}S-Labeled 3*H*-BD (**4**) was prepared starting from thiobenzoic acid (**2**) by adapting the method previously described[8] for radiosynthesis (Scheme 1). Radiolabeled thiobenzoic acid was prepared by heating thiobenzoic acid (**2**) with $^{35}S_8$. Treatment of labeled-**2** with thiosalicylic acid and sulfuric acid gave the cyclic disulfide **3**. Trifluoroperoxyacetic acid, generated *in situ* from trifluroacetic acid and hydrogen peroxide, was used to oxidize **3**, thereby providing access ^{35}S-labeled 3*H*-BD (**4**).

The ^{35}S-labeled dinucleoside phosphorothioate **11** was prepared using solid-phase phosphoramidite chemistry (Scheme 2)[9] via the intermediate solid support-bound dinucleoside phosphite triester **9**.[10,11] Controlled-pore-glass (CPG) support was functionalized with amino groups and succinoylated to give support-bound carboxylic acid **5** to access **9**. The N^{Bz}-protected deoxyadenosine derivative **6** was loaded onto the solid support using carbodiimide esterification conditions to yield **7**. Removal of the 5′-dimethoxytrityl group was accomplished using dichloroacetic acid (DCA) in dichloromethane (DCM), and the resulting alcohol was coupled to 2′-OMe-uridine phosphoramidite **8** in the presence of 5-(ethylthio)tetrazole to afford sulfurization precursor **9**. In the event, treatment of **9** with ^{35}S-3*H*-BD (**4**) installed the radiolabel on the thiophosphoryl triester [R_P,S_P]-**10**, which was isolated as a mixture of diastereomers at phosphorus. This diastereomeric mixture was carried to the target compound without separation. The dimethoxytrityl group in [R_P,S_P]-**10** was removed with DCA/DCM. Subsequent treatment with 28% NH_4OH resulted in the cleavage of the dinucleotide from the solid support and simultaneous removal of the cyanoethyl and benzoyl protecting groups

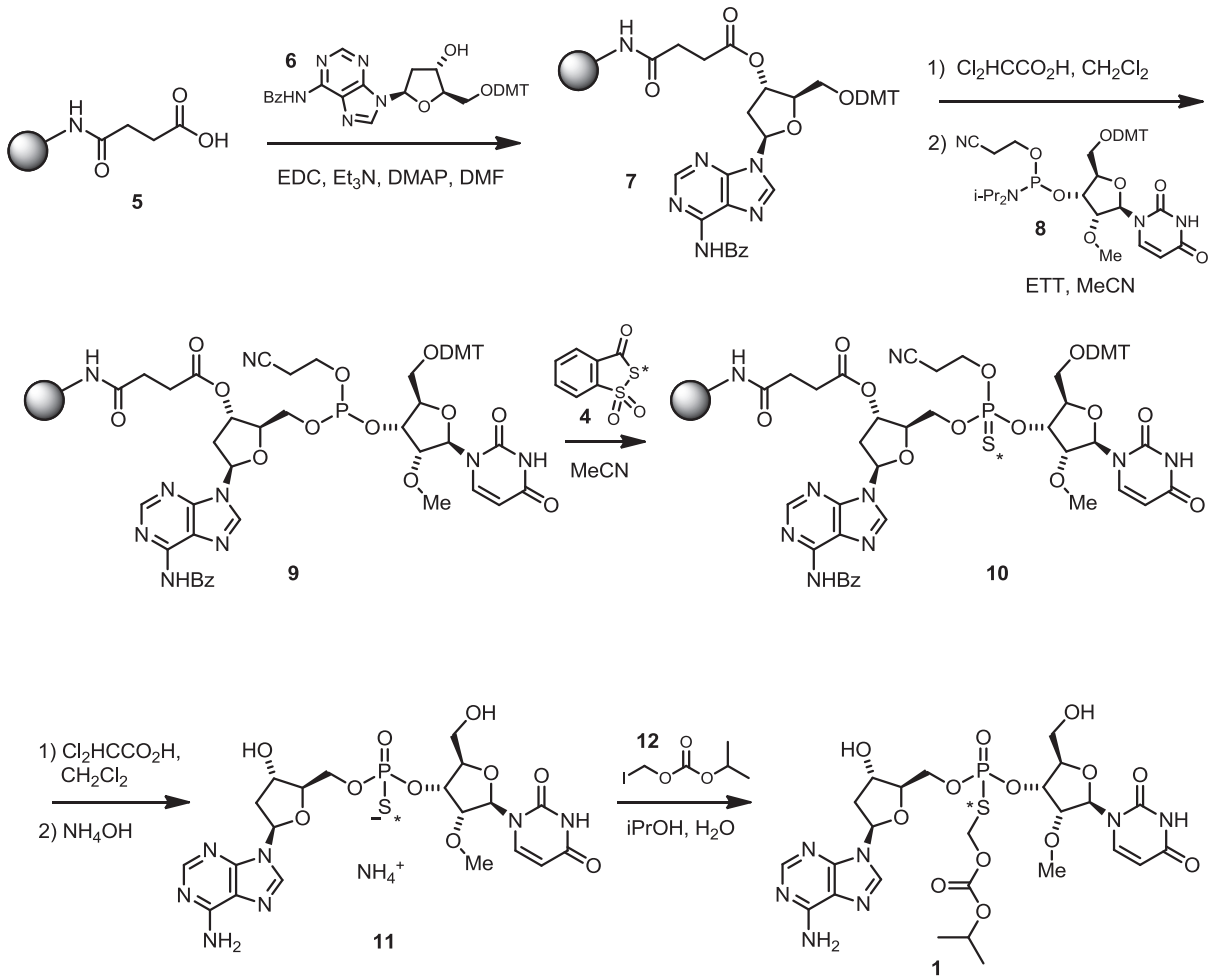
[a]*SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA*

[b]*Spring Bank Pharmaceuticals, INC., S-7, 113 Cedar Street, Milford, MA 01757, USA*

*Correspondence to: Mary J. Tanga, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA.
E-mail: mary.tanga@sri.com



Scheme 1. Synthesis of ^{35}S-3*H*-benzodithiole-3-one-1,1-dioxide (**4**).



Scheme 2. Synthesis of ^{35}S-**1** (DMT = dimethoxytrityl).

to afford the phosphorothioate [R_P,S_P]-**11**. The solution-phase alkylation of **7** with iodomethyl isopropyl carbonate (**12**) gave the target [R_P,S_P]-^{35}S-labeled **1**.

Careful work-up of the reaction mixture from alkylation proved critical to obtaining pure ^{35}S-**1**. After several attempts of the final step gave poor purity of the target compound; it was determined that ^{35}S-labeled **1** is very sensitive to high concentrations of iodide **12**. In our optimal procedure, upon consumption of **11**, as determined by analytical radio-HPLC, the isopropanol was removed *in vacuo*. Extraction with hexanes removed most of the excess iodide **12**, leaving an aqueous solution of ^{35}S-**1** and inorganic iodide salts. Application of this dilute solution directly to a preparative reversed-phase column allowed us to obtain pure ^{35}S-**1**.

Experimental

General methods

All reactions were carried out using commercial grade reagents and solvents under argon atmosphere. NMR Spectra were recorded on a Varian Mercury VMX 300-MHz spectrophotometer (Varian Medical Systems, CA, USA) using tetramethylsilane as the internal standard. NMR multiplicities are reported using the following abbreviations: s, singlet; d, doublet; m, multiplet. Low-resolution mass spectra were obtained on a Finnigan LCQDuo LC MS/MS instrument (Thermo Fisher Scientific, MA, USA) by electrospray ionization (ESI). Reversed-phase HPLC data were obtained using a Waters 2690 Separations Module (Waters Corporation, MA, USA) with photodiode array detector and a Perkin Elmer radioactivity monitor (PerkinElmer, Inc., MA, USA). Thin layer chromatography analyses were carried out on a commercial pre-coated silica gel $60F_{254}$ plates (E. Merck; 5×10 and 5×20 cm). The plates were scanned using a Bioscan System 200 Imaging Scanner (Bioscan, Inc., WA, USA). Specific activities were determined by the weighing and counting method utilizing an external standard with a liquid scintillation counter.

^{35}S-Labeled thiobenzoic acid (^{35}S-2)

Unlabeled thiobenzoic acid (**2**; 2.0 mL, 1.0 mmol, 90%), ^{35}S-elemental sulfur (0.95 mL, 95 mCi, 1 Ci/mg, Perkin Elmer Lot #04069), and toluene (7 mL) were placed in a 10-mL flask. The mixture was heated for 22 hours at 97°C. The resulting yellow mixture was cooled at room temperature (RT) and concentrated to dryness under a stream of argon. The residue was used in the next step without further purification.

*^{35}S-Labeled 3H-1,2-benzodithiol-3-one (**3**)*

Crude ^{35}S-2 was cooled in an ice bath following in which thiosalicylic acid (75.1 mg, 490 μmol) and concentrated sulfuric acid (1 mL) were added. The reaction mixture was heated for 20 hours at 50°C. The resulting dark brown mixture was cooled in a dry ice/acetone bath, and ice water (20 mL) and DCM (20 mL) were added. Additional water (20 mL) was added and the resulting mixture was extracted with DCM (3×20 mL). The organic extracts were combined and washed with 5% aqueous Na_2CO_3 (3×25 mL). The organic phase (~75 mL) was passed over a column anhydrous $MgSO_4$, and concentrated to give of a dark yellow solid (97 mg). The residue was redissolved in warm hexane, and the hexane solution was passed again through anhydrous $MgSO_4$, filtered, and the filtrate was concentrated to give ^{35}S-3*H*-1,2-benzodithiol-3-one (**3**) as a pale-yellow solid (86 mg, 50 % chemical yield based on **1**, total activity 66.63 mCi, specific activity 130 μCi/μmole).

^{35}S-Labeled 3H-1,2-benzodithiol-3-one-1,1-dioxide (^{35}S-3H-BD, 4)

^{35}S-3*H*-1,2-benzodithiol-3-one (**3**; 86 mg, 0.5 mmol, 66 mCi). The reaction flask was immersed in an ice water bath, and trifluoroacetic acid (0.6 mL) and hydrogen peroxide (0.3 mL, 30%) were added. The reaction mixture was slowly warmed to 40°C and maintained at this temperature for 4 hours. The reaction was monitored by thin layer chromatography (SiO_2, chloroform, I_2 visualization, **3**: $R_f = 0.9$, mono-oxide intermediate: $R_f = 0.6$, sulfone **4**: $R_f = 0.8$). The mixture was cooled in an ice bath, and the reaction was quenched by the addition of ice water (5 mL), producing a white precipitate. The mixture was filtered, and the precipitate was washed with cold water (3×6 mL). The solid was dried under vacuum overnight at RT to give ^{35}S-3*H*-BD (**4**; 20 mg, radiochemical yield 20.1%, total activity 13.3 mCi, specific activity 132 μCi/μmol). The compound was stored at −20°C until used.

Solid-phase synthesis

The requisite CPG-bound dinucleoside phosphite triester **6** was synthesized on a multimillimol scale using N^{Bz}-dA-loaded CPG support in conjunction with solid-phase phosphoramidite chemistry.[9] For the synthesis, we employed a specially fabricated LOTUS reactor® as previously described.[11] The N^{Bz}-dA-loaded CPG support was prepared through microwave-assisted amination and succinoylation of native CPG (500 Å) as summarized below.

Microwave-assisted amination of CPG 500

In a pressure reactor with a Teflon plug having a chemically resistant O-ring (Chemraz), CPG 500 (135 g) and 3-aminopropyltriethoxysilane (400 mL, ~3.5 mL/g) was placed. The reaction slurry was mixed well and heated in a microwave oven (800 W) for 8 minutes in 1-minute cycle with intermittent cooling. After each heating cycle, the contents of the reaction slurry was mixed well and cooled. At the end of the final heating cycle, the reaction slurry was cooled to RT and filtered. The CPG was washed with toluene (2×125 mL), MeOH (2×250 mL), $CHCl_3$ (1×250 mL), DCM (2×250 mL), and hexanes (2×250 mL). The aminated-CPG was air-dried in a glass tray and the amino loading was determined by dimethoxytrityl analysis. Typical amino loadings were found to be 90–113 μmol/g.

*Preparation of succinoylated CPG (**5**)*

In a 500-mL pressure reactor with a Teflon screw cap stopper (with a Chemraz O-ring), aminopropyl CPG (150 g) was placed followed by a solution of succinic anhydride (60 g) and 4-dimethylaminopyridine (DMAP; 7.2 g) in *N,N*-dimethylformamide (300 mL). Additional DMF (150 mL) was added to facilitate the mixing of the slurry, and the contents were heated in a microwave oven for eight cycles each of 30-second duration. ***Caution! The reaction is exothermic and adequate safety precautions should be taken in performing this step***. The resulting dark-colored reaction mixture was mixed well by shaking and cooling between the heating cycles. The completion of succinoylation was ascertained by taking a small aliquot of CPG and heating with a solution of ninhydrin in EtOH. Absence of purple color signified the completion of reaction, following which the colored slurry was filtered, and the solid was washed with DCM, MeOH, and hexanes (2×200 mL each) and dried to obtain succinoylated CPG (**5**).

Loading of nucleoside on succinoylated CPG using LOTUS reactor®: Loading of DMT-N^{Bz}-dA

For nucleoside loading of the support,[10,11] succinoylated CPG (**5**; 100 g, 103 μmol/g amino loading), DMT-N^{Bz}-dA (**6**; 19.7 g, 3 equiv., 30 mmol), and DMAP (3.66 g, 3 equiv., 30 mmol) were added to the LOTUS reactor®. Anhydrous DMF (400 mL, freshly distilled from CaH_2) was introduced and contents were mixed using an orbital shaker. Then, anhydrous Et_3N (4.2 mL, 30 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC; 5.76 g, 3 equiv., 30 mmol) were added sequentially, and the reaction mixture was mixed under orbital shaking and recycling for 8 hours. Orbital shaking was continued for 8 hours. Periodically, aliquots of recycling liquid were withdrawn and trityl analysis was carried out to determine the amount of **6** consumed. If needed, additional amount of EDC (2.95 g), DMAP (3.7 g), and Et_3N (5 mL) were added, and shaking was continued. The contents of the reactor were filtered under vacuum, and the filtrate was collected to recover the excess unreacted nucleoside. The loaded support was washed twice with MeOH, DCM, and hexanes (2×400 mL each), and the support was dried thoroughly under vacuum in the reactor. A sample of solid support isolated showed a typical nucleoside loading of 75–80 μmol/g by trityl analysis.

The nucleoside-loaded CPG was capped with CAP A and CAP B mixtures (325 mL each) for 3 hours under orbital shaking, and the capped support was filtered, washed with methanol, DCM, and finally with hexanes (2×300 mL each). The loading of dried support was typically around 70–80 μmol/g. The nucleoside-loaded support was stored at 4°C.

*Preparation of CPG-bound dinucleoside phosphite triester (**9**)*

The reaction was carried out in LOTUS reactor®.[11] Nucleoside-loaded support (**7**; 112 g, 89 mmol) was placed in the reactor and detritylated using DCA in DCM (2.5%, DCA, 3×400 mL) with DCM washes (3×400 mL) between each DCA/DCM treatment. The support was subsequently washed thoroughly with DCM (5×400 mL) and then acetonitrile (low water, <30 ppm). Detritylated nucleoside was coupled with 2′-*O*-methyluridine phosphoramidite (**8**; 5 equiv.) in the presence of 5-ethylthiotetrazole (0.4 M, 10 equiv.) in anhydrous acetonitrile. After

the removal of excess phosphoramidite by acetonitrile washings, the support was dried in the reactor at RT under a stream of argon. Support-bound **9** was removed from the reactor and stored in an argon-filled bag and stored at −20°C until ready for use in the sulfurization step during radiolabeled synthesis.

*CPG-bound ^{35}S-labeled dinucleoside O-(2-cyanoethyl) phosphorothioate (**10**)*

To a silanized[7] 50-mL flask, ^{35}S-3*H*-BD (**4**; 20 mg, 0.1 mmol, 13.3 mCi) in acetonitrile (10 mL) and CPG-bound dinucleoside phosphite triester **9** (904 mg, 0.9 mmol) were added. The reaction mixture was maintained at RT for 18 hours. The slightly reddish mixture was filtered through a sintered glass funnel (medium, 15 mL). The solid was washed with acetonitrile (3 × 10 mL) and dried *in vacuo* (~10 torr).

*^{35}S-labeled dinucleoside phosphorothioate (**11**)*

The support-bound dinucleotide **10** was treated with a solution of DCA in DCM (15 mL, 2.5% v/v). The resulting bright orange mixture was stirred for 5 minutes and then centrifuged for 5 minutes. The orange supernatant was removed carefully by pipette, and the solid was washed with DCM (10 mL). This process (DCA treatment, centrifugation, separation, and washing) was repeated three times until no orange mixture appeared. The solid was then dried *in vacuo* to afford the support-bound dimethoxytrityl deprotected compound (679 mg). It was subsequently treated with NH_4OH (3 mL, 28–30%) and maintained at RT for 22 hours. The reaction mixture was cooled on an ice bath and neutralized with careful addition of glacial AcOH (0.5 mL) to pH 6.5–7.0. The mixture was filtered through a glass sintered funnel (medium, 15 mL), and the solid was washed with water (3 × 10 mL). The filtrate and washings (~30 mL) were combined, and neutral impurities were removed by extraction with ethyl acetate (3 × 15 mL). The aqueous layer was lyophilized to give a viscous residue (428 mg), which was desalted by reversed-phase chromatography (Sep-Pak Vac 35 cc/10 g C_{18} cartridge, eluent: water → 50% acetonitrile/water gradient). Fractions containing compound **11** (monitored by radio-HPLC) were lyophilized to afford **11** as a transparent residue (32 mg, total activity 2.4 mCi).

*Iodomethyl isopropyl carbonate (**12**)*

To a solution of NaI (25 g, 166 mmol) in acetonitrile (40 mL) at RT, a solution of chloromethyl isopropyl carbonate (12.8 g, 84 mmol) in acetonitrile (15 mL) was added drop wise. The reaction vessel was covered with aluminum foil, and the mixture was maintained at RT. After 4 days, the solid was removed using filtration and washed with DCM (2 × 50 mL). The combined filtrates were concentrated. The residue was taken up in water (50 mL) and extracted with DCM (3 × 30 mL). The combined DCM extracts were washed with 5% sodium bisulfite (100 mL) and brine (100 mL), successively. The organic phase was dried over Na_2SO_4, filtered, and concentrated to give **12** as a pale-yellow oil (14.63 g, 71%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2 (6H, d), 4.8 (1H, m), 5.8 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22, 36, 76, 154; MS (ESI+) *m/z* 244.

^{35}S-**1**. In a 50-mL flask covered with aluminum foil, **11** (32 mg, 0.0525 mmol, 2.4 mCi) in 4:1 water/isopropanol (5 mL) and iodomethyl isopropyl carbonate (**12**; 40 mg, 0.163 mmol) was placed. The reaction mixture was maintained at RT in darkness. Radio-HPLC analysis after 20 hours showed unreacted **11**. Additional quantities of **12** (80 mg, 0.326 mmol) were added. After an additional 40 hours at RT, **11** was not observed by analytical radio-HPLC. Isopropanol was removed *in vacuo*, and the remaining aqueous solution (10 mL) was extracted with hexane (3 × 10 mL) to remove excess **12**. *Without further concentration*, the aqueous layer was passed through a reversed-phase column (Sep-Pak Vac 35cc, C_{18} cartridge, eluent: water → acetonitrile gradient). Fractions containing [R_P,S_P]-^{35}S-**1** (monitored by radio-HPLC) were lyophilized to give the target compound as an amorphous solid (16.5 mg, total activity 2.27 mCi, specific activity 96.4 μCi/μmol).

A second run was carried out concurrently on the same scale (from 95 mCi $^{35}S_8$) to afford additional [R_P,S_P]-^{35}S-**1** (17.7 mg, total activity 2.14 mCi, specific activity 84.9 μCi/μmol). The [R_P,S_P]-diastereomeric ratio was typically ~60:40 (ascertained by ^{31}P-NMR) when identical conditions were followed for non-radioactive synthesis of **1** from **9**.[4]

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Supporting Information

Radio-HPLC traces of 35S-1 are provided.

Conflict of Interest

The authors did not report any conflict of interest.

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