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# SYNTHESIS OF HIGH SPECIFIC ACTIVITY TRITIUM LABELED (*E*)-6-(BROMOMETHYLENE)-TETRAHYDRO-3-(1-NAPHTHALENYL)-2*H*-PYRAN-2-ONE AS A SELECTIVE PROBE FOR CALCIUM-INDEPENDENT PHOSPHOLIPASE $A_2$

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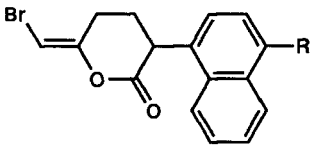
## SUMMARY

The synthesis of (*E*)-6-(bromomethylene)-tetrahydro-3-([4-$^3$H]-1-naphthalenyl)-2*H*-pyran-2-one at a specific activity of 24.0 Ci/mmol is described. This probe was synthesized to determine whether (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one inhibits calcium-independent myocardial phospholipase $A_2$ irreversibly via covalent modification. The material was synthesized in four steps from 1-naphthalene acetic acid via [4-$^3$H]-1-naphthaleneacetic acid. The yield from [4-$^3$H]-1-naphthaleneacetic acid was 29.7%. The radiochemical purity of the HPLC-purified final product was 99.5%.

**KEYWORDS:** (*E*)-6-(bromomethylene)-tetrahydro-3-([4-$^3$H]-1-naphthalenyl)-2*H*-pyran-2-one, haloenol lactones, myocardial phospholipase $A_2$, inhibition, suicide substrate.

## INTRODUCTION

A novel class of intracellular calcium-independent phospholipases $A_2$ which selectively hydrolyzes plasmalogen substrate, the major phospholipid pool in myocardium, has been isolated and characterized from canine myocardium.[1,2] A selective probe which would distinguish between calcium-independent phospholipases $A_2$ activities would prove useful in the elucidation of the physiologic and the pathologic importance of calcium-independent phospholipases $A_2$. Recently, we have discovered that the bromoenol lactone **1**, (*E*)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-




1 R = $^1$H
2 R = $^3$H

one, is >1000-fold more specific as an inhibitor of calcium-independent myocardial phospholipase $A_2$ than calcium-dependent phospholipases $A_2$.[3] The inhibition of the calcium-independent myocardial phospholipase $A_2$ by bromoenol lactone 1 was both time-dependent and irreversible, which suggested

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that compound 1 may be acting as a mechanism-based inhibitor, i.e. a suicide substrate.[3] To establish whether the bromoenol lactone 1 was a substrate which irreversibly inhibited calcium-independent canine myocardial phospholipase $A_2$ via covalent modification, we have synthesized the tritium-labeled bromoenol lactone 2, (*E*)-6-(bromomethylene)-tetrahydro-3-([4-$^3$H]-1-naphthalenyl)-2*H*-pyran-2-one at high specific activity.

## RESULTS AND DISCUSSION

Only limited quantities of purified calcium-independent canine myocardial phospholipase $A_2$ were available. Therefore, in order to accurately measure the radioactivity bound to the inactivated enzyme and to carry out required kinetic studies, it was necessary to synthesize the labeled bromoenol lactone 2 at as high a specific activity as possible. This was conveniently achieved by use of tritium reduction labeling of the halogenated naphthalene ring. The complete synthesis is outlined in Figure 1.

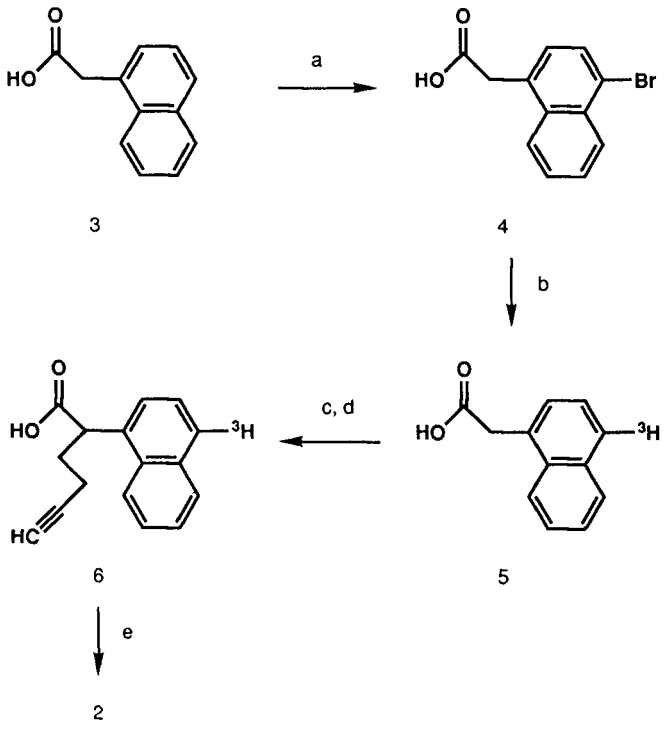


Fig. 1 Synthesis of [$^3$H]-Bromoenol lactone 2.
a: $Br_2$, $CH_3CO_2H$, 70°C; b: $^3H_2$, Pd/C, NaOAc, EtOH; c: LDA/THF;
d: $Br(CH_2)_2C{\equiv}CH$/THF; e: NBS, $KHCO_3$, $Bu_4NOH$, $CH_2Cl_2$

Bromination of 1-naphthaleneacetic acid 3 by treatment with bromine in refluxing acetic acid gave 4-bromo-1-naphthaleneacetic acid 4 in high yield.[4] This material was then test reduced with $^1H_2$ prior to reaction with tritium to optimize reduction conditions.[5] Reduction in ethanol over palladium on

activated carbon occurred rapidly, requiring 2 p.s.i. pressure of hydrogen gas for 0.5 hours. Longer reaction times or higher pressure resulted in partial reduction of the naphthalene ring. Consequently, tritium reduction under these optimized reduction conditions provided the product, [4-$^3$H]-1-naphthaleneacetic acid **5**, which was determined to be 96.3% radiochemically pure by HPLC and to have a specific activity of 23.7 Ci/mmol.

Synthesis of 1-bromo-3-butyne was accomplished by treatment of 3-butyn-1-ol with $PBr_3$.[6] The crude 1-bromo-3-butyne was treated with 1-naphthyl isocyanate to remove unreacted alcohol and then purified by distillation. Treatment of [4-$^3$H]-1-naphthalene acetic acid **5** at low temperature with two equivalents of lithium diisopropylamide in dry THF yielded[7] a bright orange solution of a dilithium salt, which on addition of 1-bromo-3-butyne afforded 2-([4-$^3$H]-1-naphthalenyl)-5-hexynoic acid **6**. Acid **6** was brominated and cyclized by treatment with N-bromosuccinimide in the presence of $KHCO_3$ and tetrabutylammonium hydroxide in $CH_2Cl_2$.[7] The product, [$^3$H]-bromoenol lactone **2**, was 66.3% radiochemically pure. It was purified by semi-preparative reverse-phase HPLC. During purification it was found necessary to (a) ensure the mobile phase of acetonitrile/water was at a neutral pH and (b) extract the product into $CH_2Cl_2$ and to dry this solvent immediately after each HPLC purification run, since the product slowly decomposed in the presence of water and this decomposition was accelerated below pH 6.

The purified product was stored in anhydrous toluene at -70°C. Radiochemical purity was found to be 99.5%. The specific activity was 24.0 Ci/mmol. After storage for 3 months, the radiochemical purity of the [$^3$H]-bromoenol lactone **2** had decreased to only 99.1%.

The tritiated bromoenol lactone **2** has been successfully utilized to demonstrate that the compound is a suicide substrate for purified calcium-independent canine myocardial phospholipase $A_2$.[3] The compound is enzymatically hydrolyzed by calcium-independent canine myocardial phospholipase $A_2$ to generate an electrophilic α-bromomethyl ketone, which covalently binds to and irreversibly inactivates the enzyme. Due to the high specificity of **1** as an inhibitor of calcium-independent phospholipase $A_2$ over calcium-dependent phospholipases $A_2$[3], the tritium labeled bromoenol lactone **2** should prove useful as a selective probe for calcium-independent phospholipase $A_2$.

## EXPERIMENTAL

All melting points were uncorrected. Reaction progress and product radiochemical purity were determined by HPLC using a Spectra-Physics SP8800 pump, a Rheodyne 7125 injector, an Altex Ultrasphere ODS 5 μm 4.6 x 250 mm column, a Kratos Spectroflow 757 detector set at 280 nm absorption or other settings as necessary, a Radiomatic Flow-One/Beta Model CR detector controlled by an IBM PC, and a Spectra-Physics SP4270 integrator. HPLC mobile phase and scintillation fluid (Flow-Scint

II, Radiomatic Instruments) were pumped at a ratio of 1:4 through the 0.5 mL cell of the Flow-One/Beta detector. Radiochemical analysis on this detector was checked by collecting fractions and examining these on an LKB 1219 Rack/Beta Liquid Scintillation Counter. Counting fluid was Insta-gel (Packard Instrument Inc.). Preparative HPLC was performed either on the above-described HPLC system using an Alltech Econosil $C_{18}$ 10 μm 10 x 250 mm semi-preparative column (for [$^3$H]-bromoenol lactone **2**) or on a Waters Prep 500A liquid chromatograph using a custom-packed PrepPAK Vydac $C_{18}$ 15-20 μm 57 x 300 mm preparative column (for 4-bromo-1-naphthaleneacetic acid **4**). NMR spectra were obtained on a Varian VXR300 FT spectrometer and chemical shifts are reported relative to TMS ($\delta$ = 0.0 ppm). Mass spectra were obtained on a Finnigan 4500 quadrupole mass spectrometer. High resolution fast atom bombardment mass spectrometry was carried out on a Finnigan MAT 90 forward geometry sector mass spectrometer. Acidity was measured by an Orion model 701A/Digital meter with a Fisher Scientific pencil combination electrode model E-5M. A sample of 1-naphthaleneacetic acid was purchased from Aldrich.

**1-Bromo-3-butyne.** A solution of $PBr_3$ (67 mL, 190 g, 0.70 mol) in ethyl ether (180 mL) was added dropwise over a period of 30 min to a stirred solution of 3-butyn-1-ol (100 g, 1.43 mol) in ethyl ether (750 mL) at 0°C in a flame-dried apparatus under dry argon. After stirring for an additional 2.5 hours at 0°C, the mixture was added to ice (1000 g) over a period of 1 hour to quench the reaction. The layers were separated and the aqueous layer was extracted with ethyl ether (1 x 250 mL). The ether layers were combined and washed with sat. $NaHCO_3$ (2 x 250 mL), $H_2O$ (2 x 250 mL), sat. NaCl (2 x 250 mL) and $H_2O$ (2 x 250 mL). The organic layer was dried (anhydrous $MgSO_4$). The ether was removed by distillation at atmospheric pressure. The residue was distilled at atmospheric pressure and the fraction with b.p. 104-5°C was collected (52.9 g). $^1$H NMR indicated the fraction contained an 86:14 molar ratio of 1-bromo-3-butyne to unreacted 3-butyn-1-ol.

A portion of the distillate (47.9 g, 0.36 mol) was treated with 1-naphthyl isocyanate (14.5 g, 0.086 mol) at 0°C with stirring under argon in an oven-dried flask to remove 3-butyn-1-ol. The mixture was stirred for 15 min at 0°C and then 15 min at room temperature. The temperature was gradually raised to 95°C and heated at that temperature for 17.5 hours. The temperature of the mixture was raised to 140°C (no higher) and 23.2 g (13.5%) of 1-bromo-3-butyne were recovered by distillation as a colorless liquid (alcohol-free and anhydrous): b.p. 109-110°C (lit. b.p.[6] 110-2°C); $^1$H NMR ($CDCl_3$) $\delta$ 2.12 (t, J = 3.3 Hz, 1 H), 2.77 (dt, J = 7.2, 3.3 Hz, 2H), 3.45 (t, J = 7.2 Hz, 2 H).

**4-Bromo-1-naphthaleneacetic acid (4).** A solution of bromine (2.9 mL, 8.5 g, 53 mmol) in glacial acetic acid (50 mL) was added to a stirred solution of **3** (10.0 g, 53.7 mmol) in glacial acetic acid (75 mL) at room temperature. The mixture was heated at 70°C for 2 hours and then allowed to sit at room temperature for 15 hours. The acetic acid was removed *in vacuo*. The residue was dissolved in $CH_2Cl_2$ and washed with $H_2O$ (3 x equal volume). The organic phase was dried (anhydrous $MgSO_4$) and the solvent was removed *in vacuo* to give 12.2 g of a white solid.

The solid was dissolved in $CH_2Cl_2$ (125 mL) and applied to an 8 x 40 cm silica column (Aldrich catalog #28,863-2, 70-230 mesh) pre-equilibrated with 10% ethyl acetate/$CHCl_3$. The column was eluted with the following solvents in the order used: 500 mL 10% ethyl acetate/$CHCl_3$, 500 mL 15% ethyl acetate/$CHCl_3$, 500 mL 20% ethyl acetate/$CHCl_3$, 500 mL 25% ethyl acetate/$CHCl_3$, 500 mL 30% ethyl acetate/$CHCl_3$, 500 mL 40% ethyl acetate/$CHCl_3$, 500 mL 50% ethyl acetate/$CHCl_3$. Nine fractions of 125 mL each (fractions 1-9) and 54 fractions of 50 mL each (fractions 10-63) were collected. Fractions were analyzed by Bakerflex silica gel IBF-2 TLC plates using 3:5:10 $H_2O$/HOAc/$CCl_4$ as developing solvent. Fractions 30-50 were combined and the solvent was removed *in vacuo* to give 8.35 g of a white solid. $^1$H NMR and analytical reverse-phase HPLC indicated that the solid was a 61:39 mixture of brominated and non-brominated **3**.

Recrystallization of the solid from HOAc/$H_2O$ provided 4.07 g (29% yield) of an 80:20 mixture of **4** and 2-bromo-1-naphthaleneacetic acid (by $^1$H NMR). A portion of the solid (850 mg) was further purified by preparative reverse-phase HPLC (0.1% aqueous acetic acid/$CH_3CN$ (50:50) at a flow rate of 50 mL/min). The collected fractions were extracted with $CH_2Cl_2$. The combined extracts were dried (anhydrous $MgSO_4$) and filtered (0.45 µm Millipore filter). The solvent was removed *in vacuo* to give **4** (660 mg) as a white solid, m.p. 171-3°C (lit.[7] m.p. 174.5-175.5°C); $^1$H NMR ($CDCl_3$) δ 4.04 (s, 2H), 7.26 (d, J = 1.8 Hz, 1H), 7.60 (m, 2H), 7.74 (d, J = 7.6 Hz, 1H), 7.94 (dd, J = 7.2, 2.4 Hz, 1H), 8.30 (dd, J = 7.2, 2.6 Hz, 1H); chemical ionization mass spectrum ($CH_4$) m/z 265/267 $(M+H)^+$, 219/221 $(M-HCOOH+H)^+$, 186 $(M-Br+H)^+$. $^1$H NMR indicated a trace amount of the 2-bromo compound was also present.

**[4-$^3$H]-1-Naphthaleneacetic acid (5).** A mixture of **4** (34.9 mg, 0.113 mmol), sodium acetate trihydrate (17.8 mg, 0.113 mmol), 5% palladium on activated carbon (12.9 mg) and ethanol (26 ml) were added to a 150 ml hydrogenation reaction vessel. The vessel was purged with nitrogen, then purged with hydrogen at 2 psi (above atmospheric). The vessel was stirred for 0.5 hours at room temperature. The reaction mixture was removed from the vessel and filtered through a 0.45 µm filter (Millipore Durapore filter unit). The solvent was evaporated and the residue dissolved in water (15 ml). The solution was acidified to pH 2 with 3N HCl. The solution was transferred to a separatory funnel and extracted three times with $CH_2Cl_2$. The combined organic layers were dried (anhydrous $MgSO_4$) and filtered. The $CH_2Cl_2$ was removed *in vacuo* giving **3** as a white solid in 98.5% purity (by HPLC using linear gradient of 0.1% aqueous acetic acid/$CH_3CN$ (50:50) to 0.1% aqueous acetic acid/$CH_3CN$ (10:90) over 20 min and a flow rate of 1 mL/min). Product identity was verified (HPLC, m.p., NMR, and mass spectrum) by comparison with an authentic unlabelled sample, m.p. 130-132°C (lit m.p. 129-131.5°C); $^1$H NMR (DMSO-$d_6$) δ 3.94 (s, 2H), 7.2-8.1 (m, 7H).

The hydrogenation was repeated on the same scale as above with tritium gas. After removal of catalyst and volatile radioactivity, the ethanol was evaporated, and the residue taken up in water. The aqueous solution was extracted and dried as described above. After filtration, the solvent was

removed *in vacuo* giving **5** as a white solid (4.71 mg, 600 mCi, specific activity 23.7 Ci/mmol, radiochemical purity 96.3%). Chemical identity of **5** was verified (HPLC and mass spectrum) by comparison with an authentic unlabelled sample.

**2-(1-[4-$^3$H]-Naphthalenyl)-5-hexynoic acid (6).** A 2 ml vial and cap with a teflon-lined silicon septum (Pierce Chemical Co), and a teflon micro-stirrer, were dried in an oven overnight. The vial was filled with dry nitrogen and capped. N-Butyllithium in hexanes (1.6 M, 35.1 µL, 56.0 µmol) followed by dry THF (30 µL) were injected into the vial through the septum. The vial was cooled to minus 20°C ($CCl_4$/solid $CO_2$) and the contents stirred. Dry diisopropylamine (7.30 µL, 52.0 µmol) was injected to form lithium diisopropylamide. Labeled 1-naphthaleneacetic acid **5** (600 mCi, 4.71 mg, 25.3 µmol), previously dried for 12 hours over $P_2O_5$ in a vacuum desiccator, was dissolved in dry THF (25 µL) and the solution was injected into the vial. A yellow-orange solution formed, which was stirred under nitrogen for 1.5 hours at 0°C. Hexamethylphosphoramide (6 µL) was added to give an orange-red solution. This was treated with 1-bromo-3-butyne (3.43 mg, 25.8 µmol) in anhydrous THF (20 µL). After addition the solution was stirred under nitrogen at 0°C for 2 hours and at room temperature for 14 hours. The reaction mixture was acidified with 1N HCl (250 µL) and extracted with ethyl ether (3 x 250 µL). The combined organic layers were back extracted with water (1 x 150 µL). The ether solution was dried over anhydrous magnesium sulfate and filtered. Removal of solvent *in vacuo* gave **6** (3.21 mg, 319.8 mCi, estimated by HPLC using 0.1% aqueous acetic acid/$CH_3CN$ (60:40) with flow rate of 1 ml/min) (53.3% radiochemical yield from **5**). The product contained approximately 0.29 mg of starting material and two other impurities. This product was not further purified or characterized and synthesis was continued on the crude material.

**(*E*)-6-(Bromomethylene)-tetrahydro-3-([4-$^3$H]-1-naphthalenyl)-2*H*-pyran-2-one ([$^3$H]-bromo-enol lactone 2).** A mixture of powdered potassium bicarbonate (1.40 mg, 14.0 µmol), N-bromosuccinimide (2.49 mg, 14.0 µmol) and **6** (3.21 mg, 319.8 mCi, 13.5 µmol) were dissolved in $CH_2Cl_2$ (0.3 mL). Tetrabutylammonium hydroxide (1.5 M (40%), 3.4 µL) was added and the solution was stirred for 3 hours in a sealed vial at room temperature. $CH_2Cl_2$ was added, and the solution was washed once each with 2/3 volumes of 5% sodium thiosulfate solution, water, brine and water. The $CH_2Cl_2$ solution was dried (anhydrous $MgSO_4$) and filtered. Removal of $CH_2Cl_2$ *in vacuo* gave crude **2** (3.88 mg, 303.3 mCi) as an oil. The product was 66.3% radiochemically pure. This material was purified by semi-preparative reverse-phase HPLC ($H_2O$/$CH_3CN$ (48:52) at a flow rate of 6.0 mL/min). After each of the four purification runs, the collected fraction was immediately extracted into $CH_2Cl_2$, which was dried (anhydrous $MgSO_4$). The dried solutions were filtered and the combined filtrates evaporated under dry nitrogen giving **2** (2.36 mg, 178.20 mCi at specific activity 24.0 Ci/mmol) (radiochemical yield from **6** 55.7%) as a colorless oil. The product was found to be 99.5% radiochemically pure by HPLC using two mobile phase conditions: 1) isocratic, 40% $H_2O$/60% $CH_3CN$ (v/v), and 2) gradient, 40% $H_2O$/60% $CH_3CN$ (v/v) to $CH_3CN$ (100%) over 20 min and hold. The product was stored in anhydrous

toluene (45 mL) at minus 70°C. Product identity was firmly established by comparison (HPLC using above two mobile phase conditions and 50% $H_2O$/50% $CH_3OH$ (v/v) mobile phase condition, and UV absorption) with authentic unlabeled material, $^1H$ NMR ($CDCl_3$) δ 2.35 (m, 2H), 2.82 (m, 2H), 4.55 (dd, J = 8.4, 6.6 Hz, 1H), 6.09 (t, J = 1.5 Hz, 1H), 7.30 (m, 1H), 7.50 (m, 3H), 7.86 (m, 3H); electron impact mass spectrum (70 eV) m/z (% relative intensity) 318/316 (100, M+), 290/288 (10, (M-CO)+), 237 (65), 209 (90); high resolution fast atom bombardment mass spectrometry utilizing the matrix m-nitrobenzyl alcohol/lithium salt $(M+Li)^+$ = 323.0241 (calculated for $C_{16}H_{13}O_2BrLi$, 323.0259). Proton nuclear Overhauser effect studies confirmed the trans (*E*) geometry of the bromide relative to the lactone oxygen on unlabeled material.[3]

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