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# A Facile Synthesis of 3,3,4,4,3',3',4',4'-Homocystine-$d_8$

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## SUMMARY

We describe the synthesis of 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2** in four steps. Compound **2** has been utilized as an internal standard for the LC-MS quantification of L-homocysteine **1**, a marker for cardiac disease. 1,1,2,2-Tetradeutero-2-bromoethyl triphenylmethyl sulfide **4** was treated with the dianion of *N*-(*tert*-butoxycarbonyl) glycine *tert*-butyl ester **5** giving the homocysteine derivative **6**. Oxidation of **6** with iodine in methanol gave the homocystine precursor **7**. Subsequent deprotection provided **2** in high chemical purity (99%) as measured by analytical HPLC and isotopic purity of >99% as determined by mass spectrometry.

**Key Words**: homocysteine, homocystine, homocystine-$d_8$, deuterium labeling

## INTRODUCTION

L-Homocysteine **1** (Figure 1) is an established marker (1-3) for vascular diseases (4-6), neonatal-homocystinuria (7), and nutritional assessment (8,9). Heart transplant patients and patients with end-stage renal disease are also monitored for elevated levels of L-homocysteine **1** (8). The clinical significance of **1** has expanded in the last few years as it has been recognized that increased homocysteine concentration in plasma is an independent, reversible risk factor for premature cardiovascular disease and is also a contributing factor in the pathogenesis of neural tube defects (10). As a



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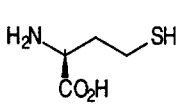
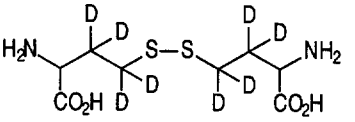
result of the clinical importance of **1**, several diagnostic methods have emerged for the accurate and rapid measurement of L-homocysteine **1** in plasma (8,9,11-19).




**1**

L-Homocysteine




**2**

3,3,4,4,3',3',4',4'-Homocystine-$d_8$

**Figure 1**

A precursor to L-homocysteine **1**, 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2**, has been utilized as a standard for the quantification of L-homocysteine **1** in plasma, serum and urine by various assays based on mass spectrometry (17-19). However, the synthesis of 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2** has not been published. Our ongoing research in this area (20,21) required **2** of high chemical and isotopic purity for the further development of an assay for L-homocysteine **1**.

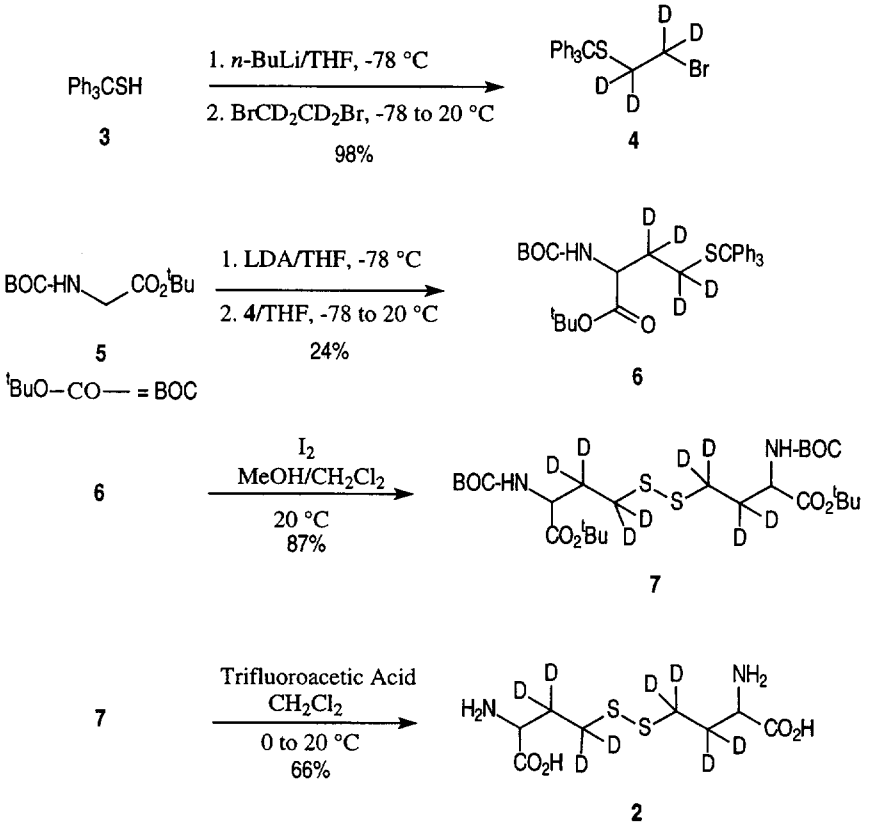
## RESULTS AND DISCUSSION

Herein, we present a four step synthesis of 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2** starting from commercially available triphenylmethyl mercaptan **3**, *N*-(*tert*-butoxycarbonyl) glycine *tert*-butyl ester **5** and 1,2-dibromoethane-$d_4$ as the deuterium source as shown in Scheme 1. 1,1,2,2-Tetradeutero-2-bromoethyl triphenylmethyl sulfide **4** was synthesized by treating triphenylmethyl lithium thiolate with excess 1,2-dibromoethane-$d_4$ at -78 °C for 2 h and at room temperature for 2 h. Reaction of bromide **4** with the dianion (22) of *N*-(*tert*-butoxycarbonyl) glycine *tert*-butyl ester **5**, generated using two equivalents of lithium diisopropylamide (LDA) at -78 °C, gave *N*-(*tert*-butoxycarbonyl)- 3,3,4,4,3',3',4',4'-homocystine-$d_8$ *tert*-butyl ester **6** as a solid in 24% isolated yield after purification by flash chromatography.

A two step process, oxidation then deprotection, was successful in producing **2** of high chemical and isotopic purity (23). The trityl group in compound **6** was removed using iodine in methanol (24) to afford the disulfide **7** in 87% yield after flash chromatography. Treatment of the homocystine precursor **7** with trifluoroacetic acid in methylene chloride gave 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2** after purification

by preparative HPLC in 66% yield as a white powder. Analysis of **2** by HPLC (25) and mass spectrometry indicated that the material produced was of high chemical and isotopic purity, 99% and >99%, respectively.




**Scheme 1**

In summary, we have developed a four step synthetic strategy for the facile preparation of 3,3,4,4,3',3',4',4'-homocystine-$d_8$ **2** from 1,2-dibromoethane-$d_4$, triphenylmethyl mercaptan **3** and *N*-(*tert*-butoxycarbonyl) glycine *tert*-butyl ester **5**. Our rapid and convenient method incorporates the deuterium labels early in the synthesis. This offers the flexibility of preparing deuterated analogs of homocysteine or homocystine.

## EXPERIMENTAL

**General.** D,L-Homocystine was purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Aldrich (Milwaukee, WI, USA) and were used as received. HPLC grade solvents were purchased from EM Science (Gibbstown, NJ, USA) and were used as received. Tetrahydrofuran was distilled over sodium benzophenone ketyl (26). $^{1}H$ (300 MHz) and $^{13}C$ NMR (75 MHz) spectra were recorded on a Varian Gemini 2300 spectrometer and reported in ppm using tetramethylsilane as the internal standard unless otherwise indicated. Electrospray mass spectra (ESMS) were obtained on a PE Sciex API 100 system. FAB HRMS was acquired on a JEOL JMS-SX102A Hybrid Mass Spectrometer and the HRFT-MS was acquired on a FTMS T-70 Finnigan NewStar System. The melting points, which are uncorrected, were obtained on an Electrothermal® melting point apparatus. Thin-layer chromatography was performed using pre-coated Whatman MK6F silica gel plates purchased from Whatman, Inc. (Clifton, NJ, USA) and visualized with UV light and/or phosphomolybdic acid reagent (20 wt% solution in ethanol). Analytical HPLC was performed on a Waters μ-Bondapak 8 × 100 mm column with λ = 225 nm and a flow rate = 1.0 mL/min. Preparative HPLC was performed on a Waters μ-Bondapak 40 × 100 mm column with λ = 225 nm and a flow rate = 20 mL/min.

***tert*-Butyl 2-[(*tert*-butoxycarbonyl)amino]-3,3,4,4-tetradeutero-4-(tritylsulfanyl)-butanoate 6.** *n*-Butyllithium (17.0 mL, 2.5 M in hexanes, 42.5 mmol, 1.2 eq.) was added dropwise to a -78 °C solution of triphenylmethyl mercaptan (10.0 g, 36.2 mmol, 1.0 eq.) in dry THF (120 mL) under a nitrogen atmosphere. After 15 min, 1,2-dibromethane-$d_4$ (10 mL, 116 mmol, 3.0 eq.) was added all at once and the yellow reaction mixture was stirred for 2 h at -78 °C and 2 h at room temperature. The yellow solution was concentrated *in vacuo* and the residual solid was partitioned between EtOAc (300 mL) and water (300 mL). The two layers were separated and the aqueous layer was extracted with EtOAc (100 mL). The combined organic layers were dried ($Na_2SO_4$), decanted and concentrated *in vacuo* to afford 1,1,2,2-tetradeutero-2-bromoethyl triphenylmethyl sulfide 4 as an off-white solid (13.45 g, 98%), mp 124-126 °C. This solid was used without further purification. $^{1}H$ NMR ($CDCl_3$) δ 7.45-7.34 (m, 6 H), 7.33-7.20 (m, 9 H). $^{13}C$ NMR ($CDCl_3$) δ 144.46 (3C), 129.48 (6C),

128.02 (6C), 126.88 (3C), 67.38 [*note*: the carbons directly connected to $^2H$, i.e. C1 and C2, were not observed].

Lithium diisopropylamide mono(tetrahydrofuran) (1.5 M in cyclohexane, 6.3 mL, 9.45 mmol, 2.2 eq.) was added dropwise to a –78 °C solution of *N*-(*tert*-butoxycarbonyl) glycine *tert*-butyl ester **5** (1.0 g, 4.3 mmol, 1.0 eq.) in dry THF (40 mL) under a nitrogen atmosphere and stirred for 30 min. A solution of **4** (2.0 g, 5.2 mmol, 1.2 eq.) in dry THF (10 mL) was added to the reaction mixture and the resulting cherry-colored solution was stirred for 5 h at -78 °C then gradually warmed to room temperature while stirring overnight. The reaction mixture was concentrated *in vacuo* and the residue was partitioned between EtOAc (100 mL) and water (100 mL). The aqueous layer was extracted with EtOAc (100 mL) and the combined organic extracts were dried ($Na_2SO_4$), decanted and concentrated *in vacuo*. Purification by flash chromatography using 10% EtOAc/hexanes gave compound **6** as a solid (0.56 g, 24%), mp 129-130 °C. $^1H$ NMR ($CDCl_3$) δ 7.41-7.37 (m, 6 H), 7.31-7.18 (m, 9 H), 4.74 (d, $J$ = 8.1 Hz, 1 H), 4.02 (d, $J$ = 7.8 Hz, 1 H), 1.40 (s, 9 H), 1.37 (s, 9 H). $^{13}C$ NMR ($CDCl_3$) δ 171.13, 155.14, 144.72 (3C), 129.54 (6C), 127.88 (6C), 126.64 (3C), 81.92, 79.58, 66.71, 53.32, 28.28 (3C), 27.89 (3C) [*note*: carbons directly connected to $^2H$, i.e. C3 and C4, were not observed]. ESMS calcd. for $(C_{32}H_{35}D_4NO_4S+NH_4)^+$ 555.7, found $(M+NH_4)^+$ 555.3. *Elem. anal.* Calcd. for $C_{32}H_{35}D_4NO_4S$: C, 71.47; H, 7.31; N, 2.60; S, 5.96. Found C, 71.64; H, 7.03; N, 2.34; S, 6.00.

**Di(*tert*-butyl) 2,2,17,17-tetramethyl-4,15-dioxo-7,7,8,8,11,11,12,12-octadeutero-3,16-dioxa-9,10-dithia-5,14-diazaoctadecane-6,13-dicarboxylate 7.** A solution of iodine (1.16 g, 4.57 mmol, 2.5 eq.) in MeOH (50 mL) was added dropwise to a solution of **6** (978 mg, 1.82 mmol, 1.0 eq.) in MeOH (11 mL) and $CH_2Cl_2$ (3 mL). The reaction mixture was stirred for 45 min at room temperature, quenched with 0.5 N aqueous $Na_2S_2O_3$ and concentrated *in vacuo*. The resulting residue was dissolved in EtOAc (50 mL) and washed with 50 mL 5% aqueous $Na_2S_2O_3$ solution. The organic layer was separated, dried ($Na_2SO_4$), decanted and concentrated *in vacuo*. This residue

was purified by flash chromatography ($R_f$=0.30; 20% EtOAc/hexanes) giving 465 mg (87%) of the disulfide 7 as a colorless oil. $^1$H NMR (major isomer, $CDCl_3$) δ 5.13 (br s, 1 H), 4.24 (d, $J$ = 7.8 Hz, 1 H), 1.47 (s, 9 H), 1.45 (s, 9 H). $^{13}$C NMR ($CDCl_3$) δ 171.20 (2C), 155.31 (2C), 82.23 (2C), 79.80 (2C), 53.03 (2C), 28.30 (6C), 27.99 (6C) [*note*: carbons directly connected to $^2$H, i.e. C7, C8, C11 and C12, were not observed]. FAB HRMS: Calcd for $C_{26}H_{40}D_8N_2O_8S_2$, 588.3354; found, 588.3355.

**3,3,4,4,3',3',4',4'-Homocystine-$d_8$ 2.** Trifluoroacetic acid (TFA, 5 mL) was added to a 0 °C solution of 7 (450 mg, 0.764 mmol) in dichloromethane ($CH_2Cl_2$, 5 mL). The cooling bath was removed and the yellow solution was stirred at ambient temperature for 1.5 h. The reaction mixture was concentrated *in vacuo*, the resulting residue was dissolved in 5% aqueous hydrochloric acid (8 mL) and purified by HPLC using 97/3 0.04% aqueous TFA/$CH_3CN$ ($R_t$ = 7.38 min) giving 214 mg (66%) of 2, a white powder, as its trifluoroacetate salt, mp 225-227 °C (dec.). $^1$H NMR ($CH_3OH$: $D_2O$ external standard, ~1:10) δ 3.22 (s). $^{13}$C NMR ($CH_3OH$:$D_2O$ external standard, ~1:10) δ 183.44, 55.52 [*note*: carbons directly connected to $^2$H, i.e. C3, C4, C3′ and C4′, were not observed]. Analytical HPLC: $R_t$=5.02 min (99%) [under these conditions commercial D,L-homocystine has a $R_t$ of 5.05 min (>98%)]. HRFT-MS: Calcd for $(C_8H_8D_8N_2O_4S_2+H)^+$, 277.1126; found, 277.1126.

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23. An attempt at direct conversion of **6** to 3,3,4,4,3′,3′,4′,4′-homocystine-$d_8$ **2** employing acidic hydrolysis to remove all protecting groups followed by air oxidation was not as successful as the two step oxidation/deprotection process, i.e. lower yield (<35% vs. 57% for the two step process) and **2** that was produced contained carbon containing impurities. A suspension of **6** (440 mg, 0.82 mmol) in 6 M HCl (8 mL) was refluxed for 6 h, cooled and extracted with $Et_2O$. The aqueous layer was made basic (pH=9), stirred for 15 h and oxidized in air with catalytic iron (III) nitrate at pH 8. The separated solid was treated with boiling water, the heterogeneous mixture was cooled, filtered and the filtrate lyophilized to afford 40 mg of a white powder. ESMS 277 $(M+H)^+$. *Elem. anal.* Calcd. for $C_8H_8D_8N_2O_4S_2$: C, 34.76, H, 5.83; N, 10.14; S, 23.20. Found C, 37.65; H, 6.56, N, 9.92, S, 19.49.
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