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Stereoselective Reduction via Lithium Borotritide: Synthesis of ^{3}H-labeled 2-Hydroxy-N-[(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexyl)methyl]-5-methylbenzamide

Douglas D. Dischino*, Nicholas A. Meanwell,
Hiromi Morimoto§, Bradley C. Pearce, Chit Than§,
Albert F. Torri, Philip G. Williams§, and Kuo-Long Yu

The Richard L. Gelb Center for Research and Development
Bristol-Myers Squibb Company
5 Research Parkway, Wallingford, CT. 06492-7660, U. S. A.
and
§The National Tritium Labelling Facility and Structural Biology Division,
Lawrence Berkeley National Laboratory, Berkeley, CA 94720, U.S.A.

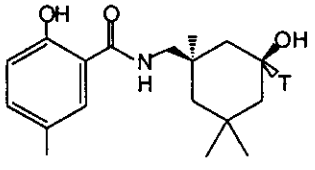
Summary

^{3}H-labeled 2-hydroxy-N-[(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexyl)methyl]-5-methylbenzamide, **1**, was prepared in one step by the reduction of the corresponding ketone with high specific activity $LiBT_4$. The reduction was stereoselective yielding the desired cis alcohol in a ratio of > 20:1. Purification of the compound was achieved on normal-phase HPLC. The radiochemical purity of the final product was greater than 99.5% and the specific activity was 29 Ci/mmol.

Keywords: Stereoselective reduction, $LiBT_4$, influenza.

Introduction

This paper describes the synthesis of ^{3}H-labeled 2-hydroxy-N-[(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexyl)methyl]-5-methylbenzamide, **1**.



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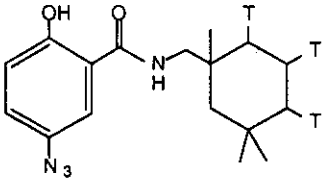
We were interested in preparing this compound since we had shown that it inhibits the action of the hemagglutinin protein of influenza virus.[1] Once labeled this compound could be used to demonstrate specific binding to the target protein, and to study the mechanism of action of certain antagonistic compounds, which we believe can bind to the target protein but do not inhibit it.

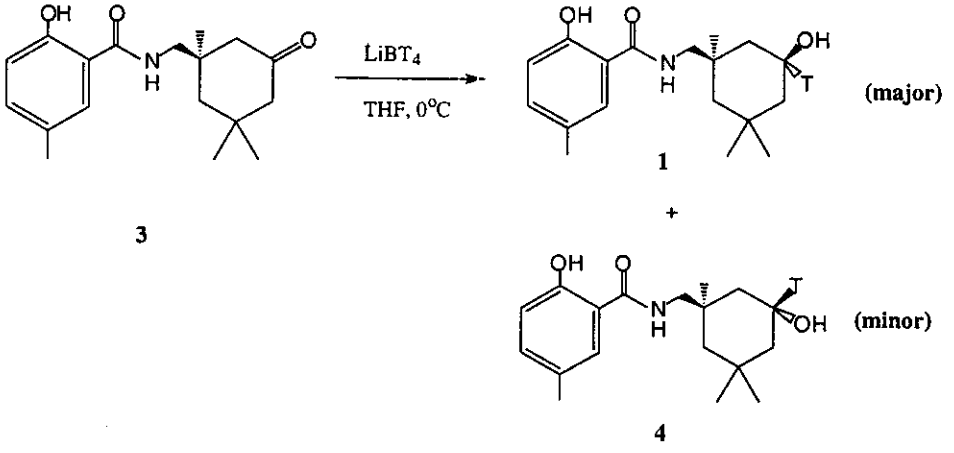
Recently we reported the synthesis of ^{3}H-labeled 2-hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, **2**, a tritium labeled photoaffinity agent for the active site of the hemagglutinin protein of influenza virus.[2,3] This compound, **2**, was prepared by reacting ^{3}H-labeled 1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methylamine with the corresponding azidosalicylic acid chloride.



2

Although we had initially planned to prepare **1** in an analogous manner, the recent development of high specific activity $LiBT_4$ prompted us to evaluate the one step reduction of **3** via $LiBT_4$ (Scheme 1). This resulted in a very efficient synthesis of our target molecule, **1**, and demonstrates the utility of high specific activity labeled $LiBT_4$ in diastereoselective reductions.

Scheme 1. Synthesis of ^{3}H-labeled 2-hydroxy-N-[(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexyl)methyl]-5-methylbenzamide, **1**.



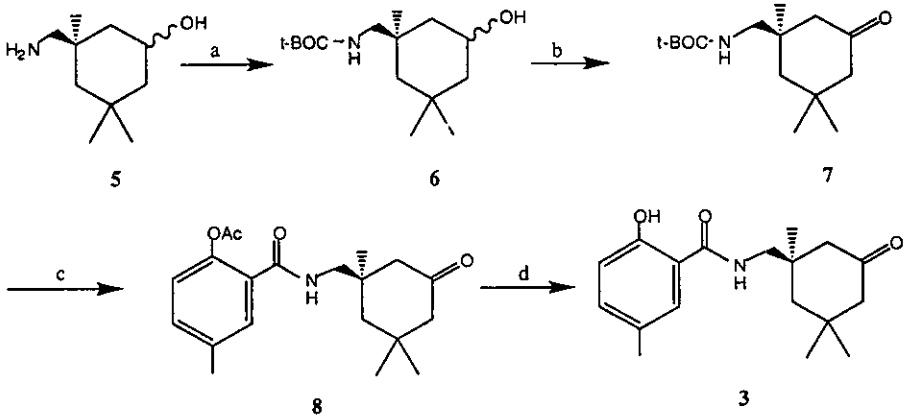
3 **1** + **4**

Results and Discussion

The synthesis of high specific activity $LiBT_4$ (110 Ci/mmol) was reported by Than and co-workers.[4,5] This new reducing agent compliments the two most frequently used tritium labeled hydrides, namely, $NaBT_4$ and $LiAlT_4$. $LiBH_4$ is often considered an analogue of $NaBH_4$, but in ether or tetrahydrofuran, the Li^+ cation is a stronger Lewis acid than the Na^+ cation, thus resulting in an increased reducing power of the lithium analogue.[6] Thus $LiBH_4$ is a stronger reducing agent than $NaBH_4$ while being more selective than $LiAlH_4$. Additionally $LiBH_4$ has been shown on many occasions to have a higher diastereoselectivity than $NaBH_4$, thereby permitting the synthesis of chiral compounds through diastereoselective reductions.[4]

Synthesis of **3** was obtained by Swern oxidation of commercially available **5**, followed by coupling to the acid chloride and subsequent removal of the protecting group via K_2CO_3/CH_3OH (Scheme 2). In the course of studying the reduction of **3** via $LiBH_4$, we observed a stereoselective reduction of **3** to **1** (desired cis alcohol) in a 2.5 to 1 ratio. These ratios were obtained by measuring the relative peak ratios of the two diastereomers on the HPLC chromatogram. Under the conditions used in HPLC Method 1, these diastereomers were separated by greater than 5 minutes. During our radioactive study, we lowered the temperature of the reaction to 0°C and obtained an enhanced stereoselectivity of 20:1 (cis:trans). Preparative normal-phase HPLC permitted isolation of the pure cis isomer in greater than 99.5% radiochemical purity. The specific activity of the compound was determined to be 29 Ci/mmol via tritium NMR analysis and HPLC.

Scheme 2. Synthesis of 2-Hydroxy-N-[(1,3,3-trimethyl-5-oxo-cyclohexyl)-methyl]-5-methylbenzamide, **3**.

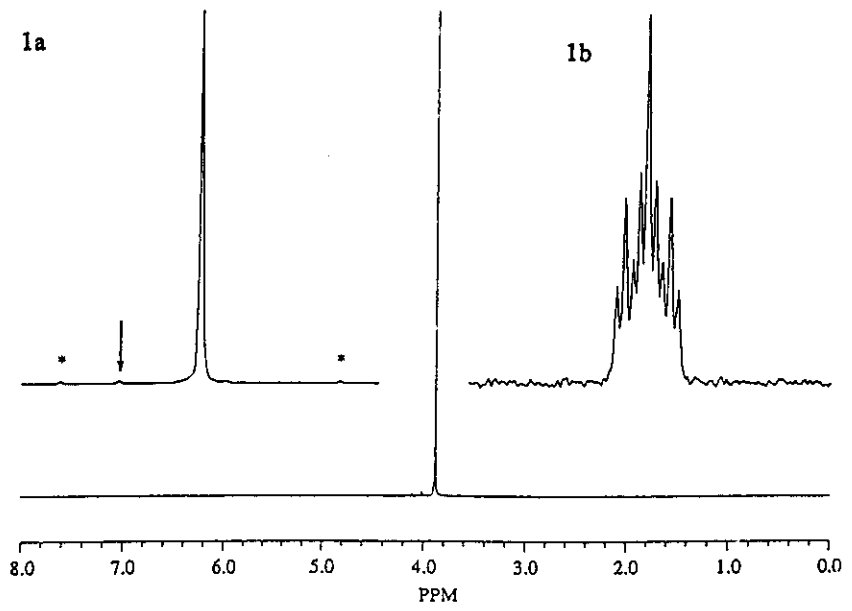


Reagents: a, Di-t-butyl dicarbonate, $NaHCO_3$, dioxane; b, ClCOCOCl, DMSO, CH_2Cl_2, -50°C, 1h, Et_3N; c, 4N HCl/dioxane, RT, 1h, DMF, ET_3N, 2-acetoxy-5-methylbenzoyl chloride, CH_2Cl_2; d, K_2CO_3/CH_3OH

Tritium NMR analysis of the HPLC purified product, **1**, confirmed the unique location of the label (Figure 1) and the extremely high chemical purity of the product. The proton decoupled ^{3}H NMR spectrum showed a single peak at 3.88 ppm, diagnostic for the cis isomer. The only other features of this spectrum are the ^{13}C satellites [J(C-T) = 148.5 Hz, 0.55% each, see expansion in Figure 1a], and another small peak at 4.01 ppm, consistent with a trace of trans isomer (~0.6% by comparison with ^{13}C satellites). The proton coupled ^{3}H spectrum in Figure 1b shows the expected complexity from coupling to four adjacent protons.

Binding studies with ^{3}H-labeled 2-hydroxy-N-[(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexyl)methyl]-5-methylbenzamide, **1**, using purified hemaglutinin (BHA) were then conducted. Initial attempts to conduct the binding studies using ultra centrifugation or equilibrium dialysis were unsuccessful. However, a filtration method using Costar Spin-X centrifuge filters was found to be effective for separating free compound from compound bound to the BHA. Using this method, a series of binding studies were conducted with **1** using a 14 point serial dilution of the BHA or of a control protein, bovine serum albumin (BSA), and the data were fitted to a binding curve to calculate binding constants. The results show that ^{3}H-labeled **1**, binds to BHA with a binding constant of 2.4 $X10^{5}$ M^{-1} and to BSA with a binding constant of 5.2 X 10^{3} M^{-1}, demonstrating that **1** specifically binds to the BHA.

Figure 1. 320 MHz Tritium NMR spectrum (proton-decoupled) of **1** in CD_3OD. 1a. Expansion of the proton-decoupled tritium NMR spectra of **1** in CD_3OD (3.6 - 4.20 ppm). The asterisks identify the ^{13}C satellites, and the arrow indicates the presence of the trans isomer. 1b. Expansion of the proton-coupled tritium NMR spectrum of **1** in CD_3OD (3.60- 4.20 ppm).



Experimental

Materials

All experimental conditions were optimized using non-radioactive materials. The identity of the final product, **1**, was confirmed by co-elution via HPLC of the radiolabeled substance with authentic unlabeled compound and by tritium and proton NMR spectroscopy. The specific activity of the final product was determined by both tritium NMR and by measuring the activity eluting from the HPLC and comparing the mass to the mass of authentic standards.

Tritium NMR Spectroscopy

Samples were made up to a volume of 250 µl in Teflon tubes (Wilmad #6005), which were then placed inside 5 mm glass NMR tubes having a screw-cap (Wilmad #507-TR-8"). NMR spectroscopy was carried out on an IBM Instrument Inc. AF-300 spectrometer (^3H at 320 MHz), using a $^3H/^1H$ 5 mm dual probe. A high quality ^3H band stop, ^1H band pass filter (Cir-Q-Tel Inc., FBT/20-300/3-6/50-3A/3A) was placed in the proton decoupling line of the instrument. Referencing of chemical shifts was achieved by generation of a ghost ^3H TMS signal from internal TMS in the ^1H NMR spectrum.

Analytical Methods

HPLC Method 1

In this method samples were loaded on a Zorbax Rx-Sil column (4.6 x 250 mm) equilibrated with 33% ethyl acetate and 67% hexane. The flowrate of the column was 1.0 ml/min. The column effluent was monitored both by uv (260 nm) and radioactivity (*IN/US* β-RAM). In this system, the ketone precursor, **3**, has a R_t of approximately 6.6 min, the undesired trans isomer, **4**, has a R_t of approximately 13.6 min, whereas, the desired cis isomer, **1**, has a R_t of approximately 19.1 min.

Purification of BHA.

Hemagglutinin (HA) was isolated from influenza virus as previously described with minor modifications.[7,8] Briefly, 50 mgs of egg purified influenza A/PR/8/34 virus (Spafas, Preston, CT) was resuspended in Buffer A (0.1 M Tris, pH 7.2, 1 mM EDTA, 50 mM β-mercaptoethanol) and added to 25 mls of Buffer B (0.1 M Tris, pH 7.5, 0.5 M NaCl) containing 2 mg/ml Bromelain (Sigma, St. Louis, MO). After incubating at 37°C for 20 h, the sample was adjusted to 0.1 M NaCl and the virus was sedimented at 140,000 X g for 3 h at 4°C. The Bromelain digested hemagglutinin (BHA) was recovered in the supernatant, concentrated using a Centriprep 30 (Amicon, Beverly, MA), and purified by gel filtration using Sephacryl 400 (Pharmacia, Piscataway, NJ) and Buffer B.

Binding Studies with ^{3}H-labeled 1.

Binding studies with ^{3}H-labeled **1** were conducted with dilutions of both BHA and Bovine Serum Albumin (BSA, Boehringer Mannheim, Indianapolis, IN) prepared in PBS. For each sample, 50 µl of protein solution was incubated with 50 µl of ^{3}H-labeled **1** (5 µM) for 10 minutes at RT, transferred to a Spin-X filter (Costar, Cambridge, MA) and centrifuged at 14,000 X g for 5 min at RT. The amount of bound and free compound at each protein concentration was then determined by scintillation measurements of the retentate and filtrate. The data from the two sets were then fitted to a binding curve ($y(x) = [(xka)/(xka+1)]$) using the Igor program (Wavemetrics, Eugene, OR) to obtain the binding constant of the compound to the proteins.

Synthesis

3-(t-Butoxycarbonylaminomethyl)-3,5,5-trimethylcyclohexanol, **6**.

To a mixture of cis and trans 3-aminomethyl-3,5,5-trimethylcyclohexanol, **5**, (10.0 g, 58.4 mmol, Aldrich) dissolved in dioxane (20 mL) was added di-t-butyl dicarbonate (14.02 g, 64.22 mmol) in saturated $NaHCO_3$ (100 mL) and the mixture allowed to stir at room temperature overnight. After 16 h, the solution was extracted with Et_2O. The combined ether extracts were dried ($MgSO_4$), and concentrated on a rotary evaporator. The residue was purified by flash chromatography (EtOAc/hexane) to give **6** (13.2 g, 83%) as an oil. M.S. m/e 289 $(M-NH_4)^+$. Anal. calc'd for $C_{15}H_{29}NO_3$: C, 66.38; H, 10.77; N, 5.16. Found: C, 66.43; H, 10.70; N, 4.98.

3-(t-Butoxycarbonylaminomethyl)-3,5,5-trimethylcyclohexanone, **7**

To a solution of oxalyl chloride (3.30 g, 26.0 mmol) in anhydrous CH_2Cl_2 (30 mL) was added dimethyl sulfoxide (4.1 g, 52.0 mmol) and the solution allowed to stir at -78°C. After 45 min, a solution of **6**, (6.35 g, 23.4 mmol) in CH_2Cl_2 (10 mL) was slowly added. The resulting mixture was allowed to stir at -50°C for 1 h. After 1 h, triethylamine (10.12 g, 100 mmol) was added slowly. After stirring for 30 min, the reaction was warmed to RT and stirred for an additional 30 min. The mixture was diluted with water (100 mL), and extracted with CH_2Cl_2. The combined extracts were dried ($MgSO_4$) and concentrated on a rotary evaporated. The residue was purified by flash chromatography (ethyl acetate/hexane = 1:10 to 1:3) to give **7** (4.61 g, 73%) as an oil which solidified on standing at RT. IR (KBr)(cm^{-1}): 3382, 1710, 1694, 1532, 1247, 1180. ^{1}H-NMR ($CDCl_3$) (ppm)1.00, (s, 3H), 1.03, (s, 3 H), 1.06 (s, 3 H), 1.43 (s, 9 H), 2.02-2.26 (m, 4 H), 2.94 (dd, J = 6.7, 13.8 Hz, 1 H), 3.04 (dd, J = 6.8, 13.8 Hz), 4.63 (bs, NH). MS m/e 270 $(M+H)^+$. Anal. calc'd for $C_{15}H_{27}NO_3$. 0.375 H_2O: C, 65.24; H, 10.13; N, 5.07. Found: C, 65.17; H, 9.80; N, 4.88.

2-Hydroxy-N-(5-oxo-1,3,3-trimethylcyclohexylmethyl)-5-methylbenzamide, 3

To 7 (0.15 g, 0.56 mmol) was added 4 mL of 4N HCl in dioxane and the solution allowed to stir at RT for 1 h. After 1 h, the solvent was removed and the residue was kept under vacuum for 1h. The residue was then dissolved in DMF (2.5 mL) and triethylamine (0.8 mL, 5.7 mmol) was added and the mixture allowed to stir at RT for 5 minutes. The solution was cooled to 0°C, and to it was added 2-acetoxy-5-methylbenzoyl chloride (0.119 g, 0.56 mmol) dissolved in CH_2Cl_2 and the solution allowed to stir for 1 h. The reaction mixture was then diluted with anhydrous ether (30 mL) and extracted with 1N HCl (10 mL), saturated $NaHCO_3$ (10 mL), saline (10 mL), dried ($MgSO_4$) and evaporated to dryness on a rotary evaporator to yield **8** which was deprotected by treatment with CH_3OH (30 mL) and K_2CO_3 (3.0 g, 21.7 mmol) for 15 min at RT. After 15 minutes, the mixture was filtered and the filtrate concentrated on a rotary evaporator. The residue was dissolved in EtOAc (25 mL) and extracted with 1N HCl (10 mL), saturated $NaHCO_3$ (10 mL), saline (10 mL), and dried ($MgSO_4$) and concentrated on a rotary evaporator. The residue was purified by flash chromatography (ethyl acetate/hexane) to yield **3** (0.056 g, 33%) as a white solid. ^{1}H-NMR ($CDCl_3$) (ppm) 1.08, (s, 3 H), 1.11 (s, 3 H), 1.12 (s, 3 H), 1.58 (d, J = 14.3 Hz, 1 H), 1.73 (d, J = 14.3 Hz, 1 H), 2.15-2.37 (m, 4 H), 3.37 (d, J = 8.9 Hz, 2 H), 6.38 (bs, 1 H), 6.91 (d, J= 8.0 Hz, 1 H), 7.13 (d, 1 H), 7.25 (d, J = 8.0 Hz, 1 H), 11.94 (bs, 1 H). MS m/e 302 $(M-H)^-$. Anal. Calc'd for $C_{18}H_{25}NO_3$: C, 71.26, H 8.31, N, 4.62. Found: C, 71.34, H, 8.39, N, 4.51.

^{3}H-labeled 2-hydroxy-N-(5-hydroxy-[5-^{3}H]-1,3,3-trimethylcyclohexylmethyl)-5-methylbenzamide, 1

To a freshly prepared sample of $LiBT_4$ (0.105 mmol, 12.1 Ci) at 0°C was added **3** (10.9 mg, 0.0357 mmol) dissolved in anhydrous THF (0.6 mL).[4] The ice bath was then removed and the solution allowed to warm to RT with stirring. After 55 minutes, the reaction was quenched by the addition of 10% HCl/MeOH (0.150 mL) causing vigorous effervescence. The resulting pale yellow liquid was then concentrated in vacuo and the residue resuspended in CH_3OH (0.5 mL) and subsequently concentrated in vacuo to yield crude **1** (approximately 700 mCi, with a radiochemical purity of 79%). A small sample of **1** was then purified via normal-phase HPLC (Method 1) to yield 50 mCi of **1**. The radiochemical purity of **1** following HPLC purification was > 99.5%. The specific activity of the material was found to be 29 Ci/mmol by ^{3}H-NMR and HPLC. ^{3}H NMR (CD_3OD, 320 MHz, proton-decoupled) (ppm) 3.87.

Acknowledgments

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U.S. Department of Energy. PGW is also an adjunct faculty member of the Department of Pharmaceutical Chemistry, University of San Francisco, California 94143-0446.

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1. In experiments done in-house, compound 1 displayed an IC_{50} value of 30 ng/mL in the H1 influenza A red blood cell hemolysis assay.

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