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Synthesis of ^{3}H-labeled 2-Hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, a Photoaffinity Analog of an Influenza Fusion Inhibitor

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Summary

Synthesis of ^{3}H-labeled 2-hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, a photoaffinity analog of an influenza fusion inhibitor, is reported. Tritiation of a mixture of N-(t-butoxycarbonyl)-1,3,3-trimethylcyclohex-4 (or 5)-enylmethylamine via T_2 and Pd/C, followed by coupling of the deprotected tritiated amine with acetyl 5-azidosalicylic acid chloride yielded the penultimate product. Subsequent deprotection and normal phase HPLC purification yielded the target compound with a radiochemical purity > 99% and a specific activity of 63 Ci/mmol.

Keywords: photoaffinity label, tritium, influenza, hemagglutinin protein

Introduction

Influenza virus is an enveloped virus which is the etiologic agent of an acute, seasonal respiratory infection.[1] The hemagglutinin protein of influenza virus is one of three viral proteins present on the surface of the virion.[2] The hemagglutinin protein is required for initiating virus infection, through first binding to sialic acid containing receptors on the surface of the host cell. After endocytosis of the virus particle, the hemagglutinin protein also promotes uncoating of the virus particle by inducing fusion of the endosomal and viral membranes.[1]

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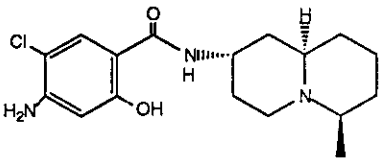
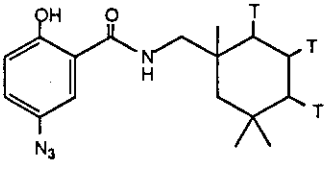
Although the mechanism which promotes the fusion of the two membranes is not fully elucidated, it is known to be induced through the action of the hemagglutinin, which undergoes an irreversible conformational change in the process.[3] The event which triggers this conformational change is the decreasing pH encountered during transport through the endosomal pathway.[3-8]

Previously, we had described a compound, BMY-27,709, which could inhibit influenza virus infection by H1 and H2 virus subtypes.[8] Initial studies showed that this compound inhibited a function of the hemagglutinin protein involved in virus-endosomal membrane fusion.[8] In addition, viruses resistant to BMY-27,709 were selected and found to have amino acid changes which map to a region of the protein believed to be involved in virus fusion.[9] Further experimentation demonstrated that the mechanism of action of BMY-27,709 is through the inhibition of the low pH induced conformational change.[9] In an attempt to better understand the mechanism of action of this class of inhibitor and to directly determine the binding pocket of these compounds within the hemagglutinin protein, we synthesized **1a**, a ^{3}H-labeled inhibitor containing a photolabile moiety.



BMY 27,709

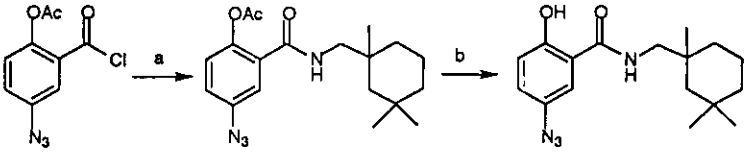


^{3}H-labeled **1a**

Results and Discussion

The synthetic chemistry and biological studies leading to the decision to prepare ^{3}H-labeled 2-hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide is the subject of another manuscript in preparation.[10] In general, the nonradioactive photoaffinity compound, **1b**, was prepared via the coupling of 2-acetyl-5-azidosalicylic acid chloride, **2**, with (1,3,3-trimethylcyclohexyl)methylamine, **3**, followed by hydrolysis of the acetate with potassium carbonate in methanol (Scheme 1).

Scheme 1. Synthesis of 2-Hydroxy-N-[(1,3,3-trimethylcyclohexyl)-methyl]-5-azidobenzamide.



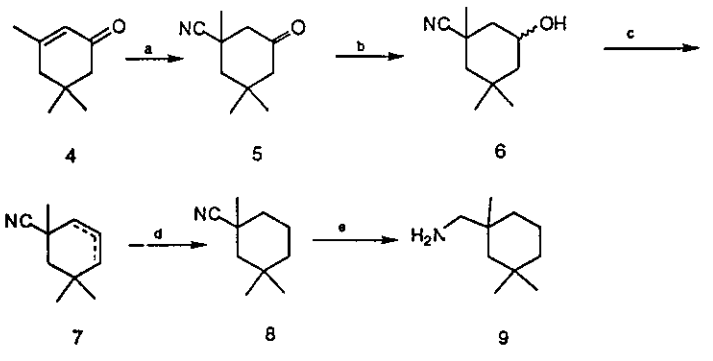
2 **3** **1b**

Reagents: a, (1,3,3-trimethylcyclohexyl)methylamine, Et_3N, DMF; b, K_2CO_3/CH_3OH.

The corresponding acid chloride was prepared by first treating 5-azidosalicylic acid,[11] with acetic anhydride and sulfuric acid, and reacting the resulting acetate with oxalyl chloride in the presence of a catalytic amount of N,N-dimethylformamide. 1,3,3-Trimethylcyclohexylmethylamine, **9**, was prepared in a multiple step synthesis from isophorone as depicted in Scheme 2. In this synthesis diethylaluminum cyanide was reacted with isophorone, **4**, followed by reduction of the ketone, **5**, with sodium borohydride to give a mixture of diastereomers of 3-cyano-3,5,5-trimethylcyclohexanol, **6**. Mesylation of the alcohols with methanesulfonyl chloride followed by elimination of methanesulfonic acid yielded a mixture of the cyanoolefins, **7**. Hydrogenation of the resulting olefins over 10% Pd/C in methanol gave the corresponding saturated nitrile, **8**, which was then reduced to the corresponding amine via lithium aluminum hydride-aluminum chloride in ether to provide **9**.

Our initial labeling strategy was the one-step reductive tritiation of the unsaturated nitrile **7** to **9** via T_2/PtO_2, followed by coupling of the tritiated methylamine, as shown in Scheme 1. This approach appeared to offer a method by which to introduce four tritium atoms into the molecule, however, no useful product was obtained from this reductive tritiation reaction and we subsequently developed an alternative approach. The successful synthesis of ^{3}H-labeled 2-hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, **1a**, (Scheme 3), involved tritiation of a mixture of N-t-(butoxycarbonyl)-1,3,3-trimethylcyclohexen-4-ylmethyl-amines and N-t-(butoxycarbonyl)-1,3,3-trimethylcyclohexen-5-ylmethyl-amines, **11**, prepared by reduction of **7** with lithium aluminum hydride-aluminum chloride, followed by protection of the unsaturated amine, **10**, with di-t-butyl dicarbonate. Deprotection of the ^{3}H-labeled amine, **12**, followed by coupling to acetyl 5-azidosalicylic acid chloride, **2**, yielded the penultimate compound **3b**. Removal of the acetyl protecting group with potassium carbonate in methanol followed by normal phase HPLC yielded the desired tritiated product, **1a** with a radiochemical purity of >99% and a specific activity of 63 Ci/mmol.

Scheme 2. Synthesis of (1,3,3-trimethylcyclohexyl)methylamine.

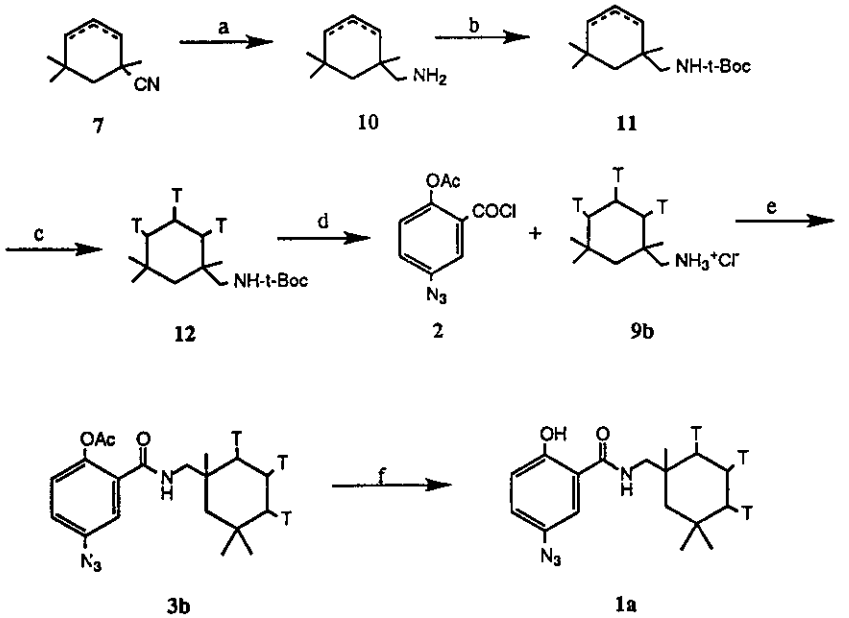


Reagents: a, $AlEt_2CN$, toluene, rt; b, $NaBH_4$, CH_3OH, 0°C; c, MsCl, Et_3N, CH_2Cl_2, 2,6-lutidine, 200°C; d, H_2, Pd/C; e, $LiAlH_4$-$AlCl_3$.

Tritium and proton NMR analyses of intermediate **12** were essential to this synthesis, since no other analytical approach proved feasible at this stage. The proton decoupled tritium NMR spectrum is shown in Figure 1, and the multiplets observed at ca. 1.50, 1.30, 1.18 and 1.12 ppm are consistent with the expected substituted cyclohexyl products.[12] The isotopomers giving rise to this spectrum may contain from one to six tritium atoms, located on any of three carbon atoms, in either axial or equatorial conformation. As a result of these many isotopomeric, positional and conformational possibilities, the tritium spectrum is a complex superposition of many spectra. Additional levels of complexity are added by ^{3}H-^{3}H couplings and isotope effects on chemical shifts in isotopomers containing multiple tritium atoms.[13]

A variety of normal phase HPLC mobile phases were evaluated for the purification of **1a**, but we were only able to achieve adequate separation of the desired compound when using an isocratic mobile phase of 30% chloroform/70% hexane. In using this system to analyze the reaction mixtures we found the reproducibility of this system was very sensitive to trace amounts of ethyl acetate. (Ethyl acetate being the extraction solvent from the last synthetic step). Thus particular care must be taken in preparing the sample for analysis. It was also observed that

Scheme 3. Synthesis of ^{3}H-labeled 2-Hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, 1a.

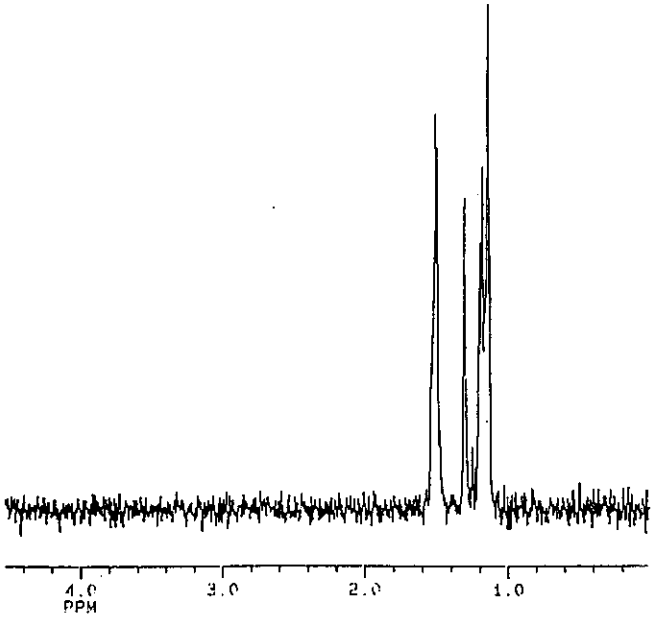


Reagents: a, LiAlH4-AlCl3, THF; b, di-t-butyldicarbonate, THF, Et_3N; c, T_2, Pd/C, EtOAc; d, 4N HCl/dioxane, 15 min, RT; e, Et_3N, DMF, CH_2Cl_2; f, K_2CO_3/CH_3OH.

during HPLC purification of **1a**, it was critical to immediately remove the mobile phase (chloroform/hexane) from the tritiated compound and then resuspend it in ethyl acetate. Failure to do so resulted in a dramatic decrease in radiochemical purity caused by radiolysis. In initial studies where **1a** had been purified by HPLC and then allowed to remain in HPLC mobile phase for only 3 h, we observed dramatic radiolysis rendering the material useless.

Affinity binding studies were then conducted with tritiated **1a**. The results from those studies and the insight they provided in understanding the mechanism of action of this class of fusion inhibitors is the subject of another manuscript.[14]

Figure 1. 320 MHz Proton-decoupled tritium NMR spectrum of **12** in CD_3OD.



Experimental

Materials

All experimental conditions were optimized using non-labeled materials. All other reagents were obtained from Aldrich Chemical Company of Milwaukee, WI and were either ACS grade or the highest quality material commercially available. The identity of the final product was established by coelution of the radiolabeled material with authentic unlabeled compound on HPLC. Radiochemical purity was determined by HPLC. The specific activity of the sample was determined by comparison of the UV absorpance of the standard with the liquid scintillation counting of the isolated HPLC peak effluent. The HPLC system consisted of Rainin

CPX pumps, a Rainin UV-1 detector for UV analysis and a *IN/US B*-RAM radioactive flowthrough detector for radioactivity measurements.

Analytical Method

HPLC Method

In this method samples were loaded on a Zorbax Rx-silica column (4.6 x 250 mm) equilibrated with 30% chloroform and 70% hexane. The flowrate of the column was 1 mL/min and the sample was monitored by both uv (260 nm) and radioactivity.

Tritium NMR Spectroscopy

Samples were made to a volume of 250 ul 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.

Synthesis

1,3,3-Trimethyl-5-oxo-cyclohexanecarbonitrile, 5.

To a solution of isophorone, 4, (27.6 g, 0.2 mol) in 50 mL of hexane was slowly added $AlEt_2CN$ (1M in toluene, 240 mL) at room temperature. After stirring for 1.5 h, the solution was cooled in ice bath, and slowly quenched with 600 mL of 2N NaOH. The resulting solution was extracted with Et_2O (350 mL, 2X). The combined extracts were washed with 1N NaOH, water, brine, dried over $MgSO_4$, and evaporated. The residue was purified by passing through a short silica gel column (CH_2Cl_2:EtOAc=1:10 to 1:4) to give a crude oil which crystallized from hexane to give 30.0 g (91%) of the product. ^1H NMR ($CDCl_3$) (ppm) 1.11 (s, 3H), 1.25 (s, 3H), 1.52 (s, 3H), 1.64 (d, J = 14.3 Hz, 1 H), 2.08 (dt, J = 1.9, 14.3 Hz, 1H), 2.16-2.31 (m, 3H), 2.70 (dt, J = 2.0, 14.5 Hz, 1H). IR (KBr, cm-1): 2975, 2232, 1715. MS m/e 164 $(M-H)^-$. Anal. Calcd. for $C_{10}H_{17}NO$: C, 72.69; H, 9.15; N, 8.48. Found: C, 72.89; H, 9.16; N, 8.47.

5-Hydroxy-1,3,3-trimethylcyclohexanecarbonitrile, 6.

To the solution of ketone 5 (30.0 g, 181.5 mmol) in methanol (150 mL) was added $NaBH_4$ (4.81 g, 127.1 mmol) in 5 portions at 0°C. After stirring at room temperature for 30 min, the reaction was cooled in a ice bath, and neutralized with conc. HCl. The solvent was evaporated. The residue was diluted with CH_2Cl_2 (100 mL), and filtered. The filtrate was evaporated, and the residue was crystallized from Et_2O-hexane to give 30 g

(99%) of the alcohol. ^{1}H NMR ($CDCl_3$) (ppm) 0.98 (s, 3H), 1.29 (s, 3H), 1.34 (d, J = 13.8 Hz, 1H), 1.44 (s, 3H), 1.49-1.58 (m, 2H), 1.67 (dd, J = 3.5 Hz, 13.8 Hz, 1H), 1.85 (d, J = 14.0 Hz, 1H), 2.02 (dd, J = 6.1, 13.8 Hz, 1H), 4.10-4.20 (m, 1H). MS m/e 168 $(M+H)^+$. Anal. Calcd. for $C_{10}H_{17}NO$: C, 71.81; H, 10.24; N, 8.37. Found: C, 72.03; H, 10.42; N, 8.30.

1,3,3-Trimethylcyclohexen-4(or 5)-ylcarbonitrile, 7.

To a solution of alcohol **6** (30 g, 179.4 mmol) and methane-sulfonyl chloride (24.6 g, 215 mmol) in CH_2Cl_2 (200 mL) was slowly added Et_3N (21.7 g, 215 mmol) at 0°C. After stirring at room temperature for 1 h, the solution was evaporated. The residue was diluted with Et_2O and washed with H_2O, dried over $MgSO_4$, and evaporated to give cis 3-cyano-3,5,5-trimethylcyclohexanol O-methanesulfonate which was used as is in the next step. ^{1}H NMR ($CDCl_3$) (ppm) 0.99 (s, 3H), 1.25 (d, J = 14.3 Hz, 1H), 1.35 (s, 3H), 1.44 (s, 3H), 1.40-1.55 (m, 2H), 1.90-2.00 (m, 2H), 2.45-2.55 (m, 1H), 3.11 (s, 3H), 5.15-5.20 (m, 1H). MS m/e 246 $(M+H)^+$. Anal. Calcd. for $C_{11}H_{19}NO_3S$: C, 53.85; H, 7.81; N, 5.71. Found: C, 53.85; H, 7.98; N, 5.56. To the 3-cyano-3,5,5-trimethylcyclohexanol O-methanesulfonate was added 2,6-lutidine (70 mL) and the mixture allowed to stir at 200°C for 1.5 h. The mixture was then cooled to -78°C, the solution diluted with water (100 mL), and acidified with conc. HCl. The mixture was extracted with Et_2O and the combined extracts were washed with water, brine, dried ($MgSO_4$), and evaporated. The residue was distilled in reduced pressure (120 mm Hg, 130-140°C) to give 13.96 g (52%) of the product as a mixture of olefins, **7**. ^{1}H NMR ($CDCl_3$) (ppm) 0.97, 1.00, 1.13, 1.25, 1.39, 1.41 (s, 9H), 1.80-1.95 (m, 3H), 2.35-2.45 (m, 1H), 5.45-5.55, 5.79-5.85 (m, 2H). MS m/e 150 $(M+H)^+$. IR: 2958, 2322, 1454 cm-1. Anal. Calcd. for $C_{10}H_{15}N$: C, 80.48; H, 10.13; N, 9.39. Found: C, 79.54; H, 10.26; N, 9.09.

1,3,3-Trimethylcyclohexen-4(or 5)-ylmethylamine, **10**

To a suspension of $LiAlH_4$ (0.5 g, 13.1 mmol) in Et_2O (100 mL) was slowly added $AlCl_3$ (1.31 g, 9.8 mL). After the suspension was stirred at room temperature for 2 h, a solution of nitrile **7** (1.3 g, 8.72 mmol) in Et_2O (5 mL) was added and the mixture allowed to stir at room temperature overnight. After 16 h, the reaction mixture was diluted with Et_2O (150 mL) and then slowly quenched with 5 N NaOH (10 mL). The suspension was then stirred at room temperature for 20 min. The organic layer was decanted and the remaining solid was washed with additional Et_2O. The combined Et_2O solutions were then dried ($MgSO_4$), and evaporated to give 1.25 g (93%) of **10** as a colorless oil. ^{13}C NMR ($CDCl_3$) (ppm) 24.8, 25.9, 28.5, 29.9, 31.0, 31.9, 32.0, 34.0, 34.8, 37.1, 38.7, 44.7, 45.6, 52.3, 53.3, 121.7, 126.9, 132.3, 136.7, 171.0, 171.6. IR (neat, cm-1) 3280, 2952, 1668, 1457. MS m/e 154 $(M+H)^+$.

N-(t-Butoxycarbonyl) 1,3,3-trimethylcyclohexen-4(or 5)-ylmethylamine, **11.**

To a solution of the **10** (1.12 g, 7.3 mmol) in THF (10 mL) was added di-t-butyl dicarbonate (1.90 g, 8.77 mmol) and Et_3N (1.48 g, 14.6 mmol). The solution was stirred at room temperature overnight. The mixture was diluted with Et_2O (100 mL), and washed with 10 % citric acid, water, brine, and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by flash chromatography (EtOAc:hexane = 1:20 to 1:10) to give 990 mg (54%) of **11**. ^1H NMR ($CDCl_3$) (ppm) 0.89-1.00 (m, 9H), 1.20-1.40 (m, 2H), 1.42 (s, 9H), 1.60-1.80 (m, 2H), 2.74-3.19 (m, 2H), 4.5 (bs, 1H), 5.28-5.50, 5.66-5.71 (m, 2H). ^{13}C NMR ($CDCl_3$) (ppm) 24.9, 26.0, 28.5, 29.9, 30.8, 31.9, 32.3, 34.2, 34.6, 36.8, 38.7, 44.9, 45.8, 50.9, 51.5, 79.1, 121.8, 126.8, 132.3, 136.7, 156.4. IR (neat, cm-1): 3357, 2955, 1704, 1510, 1173. MS m/e 254 $(M+H)^+$. Anal. Calcd. for $C_{15}H_{27}NO_2$: C, 71.10; H, 10.74; N, 5.53. Found: C, 70.76; H, 10.45; N, 5.47.

^3H-labeled N-(t-Butoxycarbonyl)-1,3,3-trimethyl-[4,5,6-^3H]cyclohexylmethylamine, **12.**

A sample of the mixture of N-(t-butoxycarbonyl)-1,3,3-trimethylcyclohexen-4-ylmethylamine and N-(t-butoxycarbonyl)-1,3,3-trimethylcyclohexen-5-ylmethylamine, **11**, (9.66 mg, 0.0382 mmol) was dissolved in EtOAc (1 mL), the solution frozen in liquid N_2, and then thawed under vacuum to degas the solution prior to tritiation. This process was repeated twice prior to the addition of the 10% Pd/C catalyst (5.3 mg). The titration was allowed to proceed under 740 mm of T_2 gas for 2h. After 2h, the tritium atmosphere was recovered in the uranium bed and the solvent removed under vacuum. Labile tritium was removed by dissolving the residue in methanol (1 mL) and evaporating under vacuum (2x). The tritium labeled product, **12** (2.2 Ci), was then dissolved in EtOAc (5 mL) and used as is in the next reaction. ^1H NMR (CD_3OD) (ppm) 0.91 (s, 6H), 0.98 (s, 3H), 1.12 (m, 2H), 1.20 (m, 2H), 1.28 (m, 2H), 1.43 (s, 9H), 1.51 (m, 2H) 2.84 (q, 2H). ^3H NMR (CD_3OD, 320 MHz, proton-decoupled) (ppm) 1.12 (m), 1.18(m), 1.30 (m), 1.50 (m).

^3H-labeled 1,3,3-trimethyl-[4,5,6-^3H]cyclohexylmethylamine, **9b.**

A 0.35 mL aliquot of **12** (150 mCi) was placed in a 25 mL pear shaped flask. To this was added fresh 4 N HCl in dioxane (0.5 mL) and the solution allowed to stir at RT for 15 min. After 15 min, the solvent was evaporated under a N_2 stream and residual solvent removed under vacuum. The residue was resuspended in acetonitrile and transferred to a 25 ml amber pear shaped flask and evaporated to dryness (2X) to yield **9 b** (150 mCi) which was used directly in the next reaction.

^{3}H-labeled 2-Hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azidobenzamide, **1a**.

To a solution of **9b** in DMF (0.5 mL) was added triethylamine (150 µl), and the solution stirred at RT for 5 min and then cooled to -78°C. To the cooled solution of **9b** was then added a solution of freshly prepared acid chloride **2**,[15] and the reaction mixture allowed to stir at -78°C for 1h, allowed to warm to 0°C over the next 30 min and then finally allowed to warm to RT for 10 min. The reaction mixture was then diluted with anhydrous ether (30 mL) and extract with 1N HCl (10 mL), saturated $NaHCO_3$ (3-4 mL), saline (10 mL), dried ($MgSO_4$) and evaporated to dryness under a N_2 stream. The residue, **3b**, was dissolved in methanol (3-4 mL) and to this was added potasssium carbonate (0.3 g) and the suspension allowed to stir for 15 min. After 15 min., the suspension was filter through a cotton plug, and the plug rinsed with methanol (1-2 mL). The organic layer was evaporated to dryness under a N_2 stream. The residue dissolved in ethyl acetate (25 mL) and extracted with 1 N HCl (10 mL), saturated $NaHCO_3$ (3-4 mL), saline (10 mL), dried ($MgSO_4$) and concentrated under a stream of N_2 to yield 87 mCi of crude ^{3}H-labeled **1a**. The radiochemical purity of **1a** was 60% (HPLC Method 1). In this system ^{3}H labeled 2-hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H]cyclohexyl)methyl]-5-azido-benzamide, 1a, has a retention time of approximately 7.8 minutes.

HPLC Purification of ^{3}H labeled 2-Hydroxy-N-[(1,3,3-trimethyl-[4,5,6-^{3}H] cyclohexyl)methyl]-5-azidobenzamide, **1a**.

An aliquot of crude **1a** (14 mCi) dissolved in ethyl acetate was concentrated to dryness under a stream of N_2 and then dissolved in mobile phase (30% chloroform/70% hexane, 0.1 mL). (It is important that all of the ethyl acetate be removed prior to HPLC purification). The compound was then injected onto the Zorbax Rx-silica HPLC column and the desired product collected. Immediately (within 15 seconds) after collection of the desired compound from the HPLC, the sample was concentrated in vacuo and then resuspended in ethyl acetate to yield 6 mCi of **1a** with a radiochemical purity of >99% and a specific activity of 63 Ci/mmol.

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15. The preparation of this acid chloride was conducted in an amber flask under reduced lighting. In a typical preparation of the acid chloride, the corresponding azido acid (17.1 mg, 0.080 mmol) was dissolved methylene chloride (0.4 mL) and to this solution was added DMF (4 µl) and oxalyl chloride (18 µl) and the reaction allowed to stir in a sealed flask for 2h at 0°C. After 2h, the solution was evaporated to dryness under a nitrogen stream and the residue resuspended in methylene chloride (0.5 mL) and used immediately.

