Synthesis and Activity of C11-Modified Wortmannin Probes for PI3 Kinase

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The key role played by PI3 kinase in cancer, hormone action, and a host of other biological functions suggests that specific inhibitors whose disposition could be ascertained in vivo would be useful in biological research or, potentially, for imaging PI3K in a clinical setting. Wortmannin (Wm, **1**) is an inhibitor of PI3 kinase with high specificity for this enzyme. We synthesized three modified Wm probes, a biotinylated Wm (**7a**), a 4-hydroxy-3-iodophenylated Wm, which was obtained both unlabeled (**7b**) and labeled with ^{125}I (**8**), and a fluoresceinated Wm (**7c**), through modification at C-11, and evaluated their inhibitive activity as inhibitors of PI3 kinase. Biotinylated (**7a**) and 4-hydroxy-3-iodophenylated Wm's (**7b**) had IC_{50}s for PI3K of 6.11 and 11.02 nM, respectively, compared to an IC_{50} for Wm of 1.63 nM. Fluoresceinated Wm (**7c**) lost considerably more activity than the other derivatives, with an IC_{50} of 64.9 nM. The ^{125}I labeled 4-hydroxy-3-iodophenylated Wm (**8**) could be detected after reaction with an immunoprecipitate of PI3 kinase. The activity of these reporter Wm's is discussed in relationship to earlier findings on the pharmacological activity of Wm derivatives and the ability of inhibitors to fit into the ATP pocket of PI3 kinase.

INTRODUCTION

Specific inhibitors of enzymes whose position can be monitored in cells have been widely used to elucidate the role an enzyme plays in biological processes (*1*−*5*). The key role played by PI3 kinase in cancer (*6*−*8*), hormone action (*9*, *10*), and a host of other biological functions (*11*−*14*) suggests that specific inhibitors, whose disposition could be ascertained, would be useful in biological or pharmaceutical research or, potentially, for imaging PI3 kinase in a clinical setting. Wortmannin (Wm) is a fungal product that is a specific inhibitor of PI3 kinase which can covalently react with a lysine in the ATP binding site of the enzyme (Figure 1) (*15*−*17*). Wm consists of a steroid-like ring system with two features that make the synthesis of Wm derivatives a challenging task. First, the furan ring, subject to attack by the epsilon amino group of lysine in the active site of PI3 kinase, can react with other nucleophiles (*18*), and this can occur in an unintended fashion. Second, the six-membered lactone ring of Wm, roughly comparable to the A ring of steroid ring system (Figure 1), is extremely sensitive to base (*19*).

Our goal was therefore to overcome these challenging features of Wm chemistry and synthesize Wm derivatives modified at C11 that might serve as "reporter Wm's," that is compounds that retained the ability of the parent Wm to inhibit PI3 kinase but with the potential to detect their position after application to a biological system. Three approaches to reporter Wm's were investigated: (i) biotinylated Wm (**7a**) was synthesized that might be detected by the Western blot method; (ii) a 4-hydroxy-3-iodophenyl Wm (**7b**) was synthesized, with nonradioactive iodine for enzyme inhibition studies and as an ^{125}I labeled (**8**) compound for autoradiography, and (iii) a fluorescent Wm (**7c**) was synthesized, for detection by fluorescence microscopy. All were made by selective modification at C-11 of Wm that had previously been shown to have little effect on the ability of Wm to inhibit PI3K (*20*). Our results show that biotinylated (**7a**) and 4-hydroxy-3-iodophenylated (**7b**) Wm's retained a good inhibitory activity for PI3K compared to the parent, but the inhibitory activity of fluoresceinated Wm (**7c**) was reduced. The relationship of these findings to earlier pharmacological and crystallographic studies of Wm's interaction with PI3 kinase are discussed.

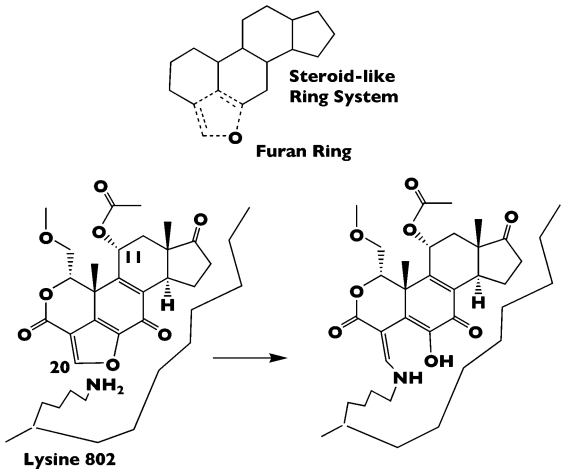

Figure 1. Wortmannin structure and reaction with PI3 kinase. Wortmannin is a steroid-like compound with a furan ring (top) that reacts with a lysine in the active site of PI3 Kinase (bottom). The positions of C-11 and C-20 on wortmannin are shown.

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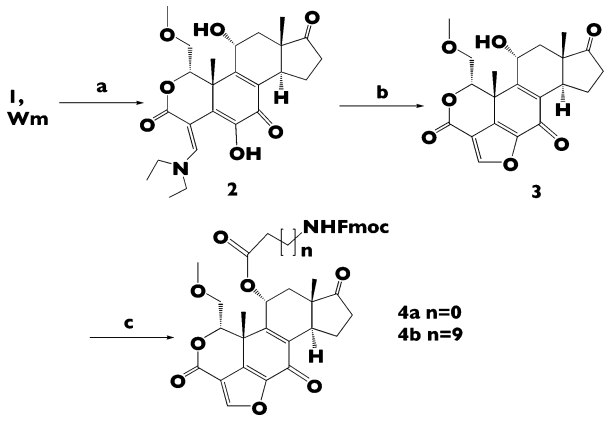
Scheme 1. Synthesis of Intermediates Used To Obtain C11-Modified Wm Probes[a]



[a] Reagents and conditions: (a) **1**, diethylamine, methanol, room temperature, 20 h; (b) 1,4-dioxane, 1 N HCl, room temperature, 22 h (75% based on wortmannin); (c) *N*-Fmoc glycine chloride (for **4a**) or *N*-Fmoc-11-aminoundecanoyl chloride (for **4b**), pyridine, dichloromethane, room temperature, overnight, (94.6% for **4a**, 89.0% for **4b**).

EXPERIMENTAL METHODS

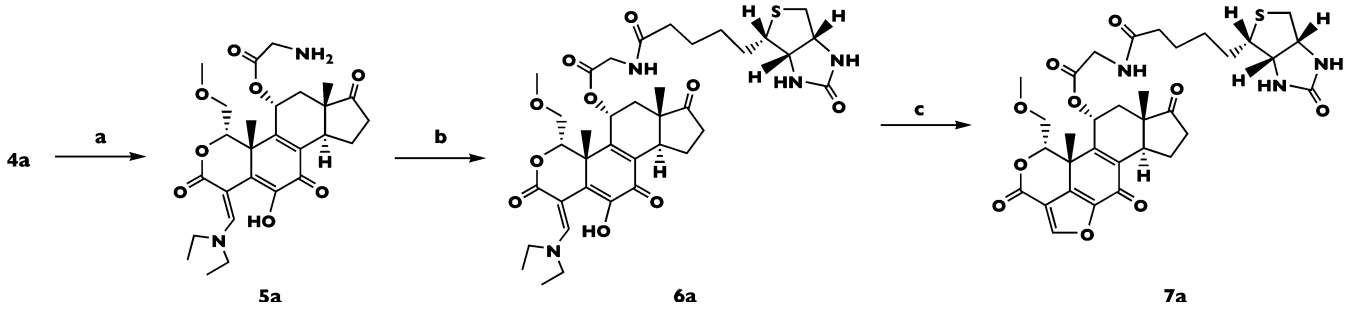
General. Wortmannin was a gift of the Natural Products Branch of the NCI. All reagents (Aldrich, or NovaBiochem) and solvents (Aldrich) were of standard quality and used without further purification unless indicated. Acetonitrile for HPLC analysis was obtained as HPLC grade from Fisher Scientific. Silica gel for column chromatography was conducted with 60−200 mesh silica gel (J. T. Baker Inc.). FITC was from Molecular Biosciences. Iodogen was purchased from Pierce and $Na^{125}I$ was from Amersham Bioscience. NMR was performed on a Varian 400 MHz instrument with $CDCl_3$ as solvent, while mass spectra were obtained on a Micromass LCT instrument with time-of-flight ESI technique. HPLC separations were accomplished on a Varian ProStar 210 gradient pumping system with a variable-wavelength PDA 330. HPLC separations were carried out on reversed-phase C18 column (VYDAC, Millipore) with water (0.1% trifluoroacetic acid,) (buffer A) and acetonitrile (containing 20% buffer A) (buffer B) as elution buffer. Three variations of this were employed. *System 1*: buffer A/buffer B (80:20) for 5 min, then linear gradient of buffer A/buffer B (80:20−0:100) over 30 min and then isocratic for 5 min, flow: 6.0 mL/min, λ_{max}: 255 nm; *System 2*: buffer A/buffer B (50:50) for 5 min, linear gradient of buffer A/buffer B (50:50−25:75) over 25 min and then isocratic for 5 min, flow: 4.9 mL/min, λ_{max}: 255 nm. *System 3*: buffer A/buffer B (70:30) for 5 min, linear gradient of buffer A/buffer B (70:30−0:100) over 45 min and then isocratic for 5 min, flow: 4.9 mL/min, λ_{max}: 440 nm.

The C11-modified Wm's were synthesized in two steps. First the Fmoc-protected intermediates **4a** or **4b** were synthesized, which were stable when stored at −20 °C for at least 3 months (Scheme 1). In a second step, the Fmoc group was removed, and the amino group was modified by the attachment of biotin (**7a**, Scheme 2), 4-hydroxy-3-iodophenylacetic acid (**7b**, Scheme 3), or FITC (**7c**, Scheme 4). The potency of the three C11-modified Wm probes as inhibitors for PI3 K was then ascertained. We also synthesized a ^{125}I version of C11 4-hydroxy-3-iodophenyl Wm (**8**) as shown in Scheme 5.

Synthesis of Intermediates 4a or 4b. *Steps a and b, Scheme 1*, were accomplished by slight modifications of published procedures (*20*). Wm (0.107 g, 0.25 mmol) was dissolved in anhydrous methanol (6 mL) with diethylamine (0.1 mL) and the furan ring opened within 10 min as indicated by the yellowish color appearance. A second portion of diethylamine (0.5 mL) was added for desacetylation of C-11 at room temperature for 20 h. After removal of methanol and diethylamine by evaporation under reduced pressure, the residue was dissolved in 1,4-dioxane (10 mL) and the furan ring closed to obtain compound (**3**). This was accomplished with 1.6 mL of aqueous 1 N HCl at room temperature in 22 h. Compound **3** was purified by silica gel column chromatography eluting with a gradient ethyl acetate/hexane with a yield of 75%. *Step c, Scheme 1*. The acylation of the C11-hydroxy group was accomplished by dissolving **3** (0.077 g, 0.2 mmol) in dichloromethane (2 mL) and anhydrous pyridine (1 mL) and using a 4-fold excess of either *N*-Fmoc glycine chloride (0.252 g, 0.8 mmol, for **4a**) or a 3-fold excess of *N*-Fmoc-11-amino-undecanoyl chloride (0.264 g, 0.6 mmol for **4b**), stirring at room temperature for overnight. **4a** and **4b** were purified by silica gel column chromatography as above. A sticky solid was obtained after the solvent was removed by evaporation. Both products were obtained by the addition of sufficient hexane to the dichloromethane solution to induce precipitation and were off-white solids for long-term storage (**4a**: 0.126 g, 94.6% and **4b**: 0.14 g, 89.0%). The structures of **4a** and **4b** were identified by NMR and HR-MS spectrometry. Selected data: **4a**: HR-MS: $C_{38}H_{35}NO_{10}$, calcd 666.2339 ($M + H^+$), found: 666.2342; ^1H NMR ($CDCl_3$, 400 MHz): 0.95 (3H, s, C*H*$_3$-13), 1.50−1.60 (1H, m, *H-12*), 1.74 (3H, s, C*H*$_3$-10), 2.01−2.07(1H, m, *H-16*), 2.20−2.30 (1H, m, *H-15*), 2.56−2.66 (2H, m, *H-12*, *H-16*), 2.81−2.88 (2H, m, *H-14*, OC*H*$_2$), 3.13 (3H, s, C*H*$_3$O), 3.14−3.21 (1H, m, *H-15*), 3.39 (1H, d, $J = 12$ Hz, OC*H*$_2$), 3.84, 3.89 (1H, dd, $J_1 = 6$ Hz, $J_2 = 18.4$ Hz, NHC*H*$_2$CO), 4.13, 4.18(1H, dd, $J_1 = 6$ Hz, $J_2 = 18.4$ Hz, NHC*H*$_2$CO), 4.22 (1H, t, $J = 6.4$ Hz, COOCH$_2$C*H*), 4.48 (2H, d, $J = 6.4$Hz, COOC*H*$_2$CH), 4.72(d, $J = 7.2$ Hz, *H-1*), 5.63 (1H, t, $J = 6$ Hz, N*H*), 6.21(1H, dt, $J_1 = 8.4$ Hz, $J_2 = 2.8$ Hz, *H-11*), 7.27−7.41 (4H, m), 7.58 (2H, t, $J = 7.6$ Hz, Ar*H*), 7.74 (1H, dd, $J_1 = 3.2$ Hz, $J_2 = 7.2$ Hz, Ar*H*), 8.28 (1H, s, *H-21*); ^{13}C NMR ($CDCl_3$, 100 MHz): 14.5, 22.9, 26.5, 35.7, 35.0, 40.7, 43.0, 44.0, 47.1, 49.2, 59.6, 67.0, 71.2, 72.9, 88.8, 114.1, 120.0, 124.8, 124.9, 127.0, 127.1, 127.7, 127.7, 140.9, 141.3, 141.4, 142.6, 143.7, 144.8, 148.8, 150.2, 156.4, 157.4, 169.0, 172.5, 215.9. **4b**: HR-MS: $C_{47}H_{53}NO_{10}$, calcd 792.3747 ($M + H^+$), found: 792.3746. ^1H NMR($CDCl_3$, 400 MHz): 0.98 (3H, s, C*H*$_3$-13), 1.28−1.67 (17H, m, OOCCH$_2$(C*H*$_2$)$_8$CH$_2$NH, *H-12*), 1.73 (3H, s, C*H*$_3$-10), 1.99−2.14 (1H, m, *H-16*), 2.20−2.31 (1H, m, *H-15*), 2.33−2.42 (2H, m, OOCC*H*$_2$), 2.56−2.63 (2H, m, *H-12*, *H-16*), 2.86−2.98 (1H, m, *H-14*), 3.00−3.12 (1H, dd, $J_1 = 6.8$ Hz, $J_2 = 11.2$ Hz, CH$_3$OC*H*$_2$), 3.17 (6H, b, OC*H*$_3$, NHC*H*$_2$, *H-15*), 3.45 (1H, dd, $J_1 = 11.2$ Hz, $J_2 = 2$ Hz, CH$_3$OC*H*$_2$), 4.22 (1H, t, $J = 6.8$ Hz, OCH$_2$C*H*Ar), 4.40 (2H, d, $J = 6.8$ Hz, OC*H*$_2$CHAr), 4.76 (1H, dd, $J_1 = 6.8$ Hz, $J_2 = 2$ Hz, *H-1*), 4.81 (1H, b, N*H*), 6.18 (1H, dt, $J_1 = 9.2$ Hz, $J_2 = 2.4$ Hz, *H-11*), 7.31 (2H, t, $J = 7.2$ Hz, Ar*H*), 7.40 (2H, t, $J = 7.2$ Hz, Ar*H*), 7.60 (2H, d, $J = 7.2$ Hz, Ar*H*), 7.77 (2H, d, $J = 7.2$ Hz, Ar*H*), 8.24 (1H, s, *H-21*); ^{13}C NMR: 14.6, 22.9, 24.7, 26.5, 26.7, 29.0, 29.2, 29.2, 29.3, 29.4, 30.0, 34.4, 35.7, 36.1, 40.7, 41.1, 44.1, 47.3, 49.2, 59.4, 66.5, 69.9, 72.8, 88.5, 114.3, 120.0, 125.0, 127.0, 127.6, 140.2, 141.3, 142.9, 144.0, 144.8, 149.7, 150.0, 156.4, 157.7, 172.2, 172.7, 216.3.

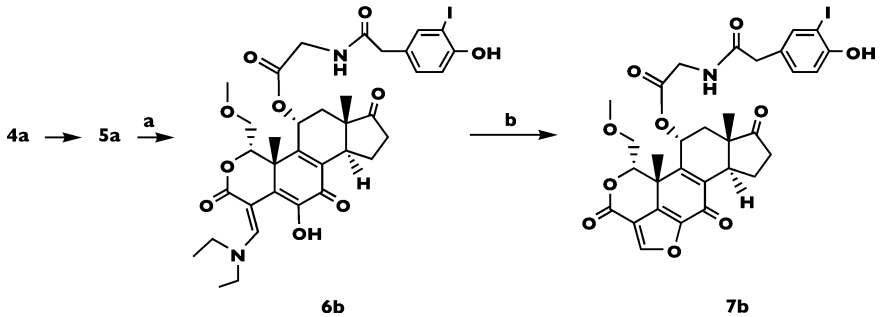
Synthesis of C11 Biotinylated Wm (7a): *For step a, Scheme 2*, a solution of **4a** (6.7 mg, 0.01 mmol) in anhydrous dichloromethane (1 mL) was added diethylamine (0.2 mL, 1.9 mmol). After stirring for 6 h at room

Scheme 2. Synthesis of C11 Biotinylated Wm (7a)[a]



[a] Reagents and conditions: (a) **4a**, diethylamine, dichloromethane, room temperature, 6 h (58.2% for **5a**); (b) **5a**, biotin, HATU, HOBT, DMF, CH_2Cl_2, r. t.; (c) 1 N HCl, DMF, 1,4-dioxane, 48 h (total yield 28.5%, based on 41.9 μmol of **4a**).

Scheme 3. Synthesis of C11 4-Hydroxy-3-iodophenyl Wm (7b)[a]

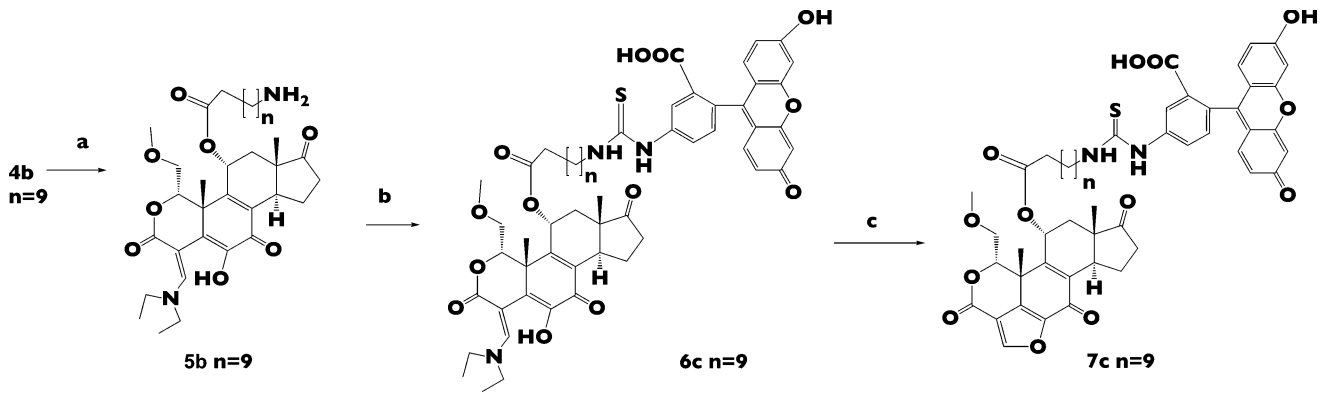


[a] Reagents and conditions: (a) **5a**, EDC, 4-hydroxy-3-iodophenylacetic acid, room temperature, 20 min; (b) 1,4-dioxane, 1 N HCl, 24 h (17.3%, overall, 0.01 mmol **4a** based).

temperature, a single major spot was obtained by silica gel TLC (CH_2Cl_2:MeOH/100:5). The solution containing **5a** was extracted with an acidic aqueous phase (3 × 5 mL 1 N HCl) and followed by the removal of impurities in the aqueous phase with dichloromethane (2 × 2 mL) extraction. The aqueous layer was then brought to pH 8 with $NaHCO_3$ powder, followed by extraction of **5a** into dichloromethane (3 × 5 mL). The solution was then dried over Na_2SO_4, and the volume was reduced to 0.5 mL with N_2 stream. Compound **5a** was characterized by HR-MS: $C_{27}H_{36}N_2O_8$, calcd 517.2550 ($M + H^+$), found 517.2552. *For step b, Scheme 2*, HATU (24.3 mg, 0.0639 mmol) and biotin (15.4 mg, 0.0633 mmol) were dissolved in anhydrous dichloromethane (3 mL) and DMF (2 mL), and the mixture was stirred at room temperature for 20 min. To this was added **5a** dissolved in 0.5 mL of anhydrous dichloromethane (0.0419 mmol based on **4a**). The mixture was stirred for 72 h at room temperature. *For step c, Scheme 2*, for the closure of the furan ring, dichloromethane was removed by evaporation and 1,4-dioxane (2 mL) was added to the remaining DMF solution. This was followed by addition of 1 N HCl in water (5 mL), and the mixture, which consisted of a single phase, was stirred at room temperature for 48 h. The mixture was extracted with dichloromethane (4 × 5 mL), and the organic layer was washed by water (2 × 5 mL). The organic layer was then dried over Na_2SO_4, and the volume was reduced by vacuum, followed by a final purification with RP-HPLC by System 1. An off-white solid was obtained by precipitation with hexane from a solution of dichloromethane. (8 mg, 28.5% overall, 0.0419 mmol based on **4a**). Selected data for compound **7a**: HR-MS: $C_{33}H_{39}N_3O_{10}S$, calcd 670.2434 (M + 1), found: 670.2435. ^{1}H NMR ($CDCl_3$, ppm): 0.96 (3H, s, C*H*$_3$-13), 1.26 (2H, m, $COCH_2CH_2CH_2CH_2CH_2$), 1.63−1.75 (8H, m, CH_3-13, H-12, $COCH_2CH_2CH_2CH_2CH_2$), 2.01−2.10 (1H, m, H-16), 2.18−2.38 (3H, H-15, CH_2CO), 2.52−2.68 (2H, m, H-12, H-16), 2.76−2.79 (1H, d, J = 12 Hz, SCH_2), 2.86−2.98 (2H, m, H-14, SCH_2), 2.95−3.08 (1H, b, SC*H*), 3.19 (5H, b, OCH_3, OCH_2, H-15), 3.50 (1H, d, J = 7.2 Hz, OCH_2), 3.92−4.13 (2H, m, COCH_2NH), 4.42 (1H, b, NHC*H*CH), 4.61 (1H, b, H-12), 5.02 (1H, b, NHC*H*CH_2), 6.00−6.20 (2H, b, NH, H-11), 6.54 (1H, b, NH), 7.08 (1H, b, NH), 8.27 (1H, s, H-21). ^{13}C NMR ($CDCl_3$, ppm): 14.8, 23.2, 25.5, 26.6, 28.1, 31.2, 35.2, 36.0, 40.6, 41.0, 41.9, 44.2, 49.4, 55.6, 59.8, 60.8, 62.2, 71.8, 73.2, 88.7, 114.5, 140.8, 143.2, 145.0, 149.2, 150.4, 158.0, 164.7, 169.6, 172.8, 174.3, 216.7.

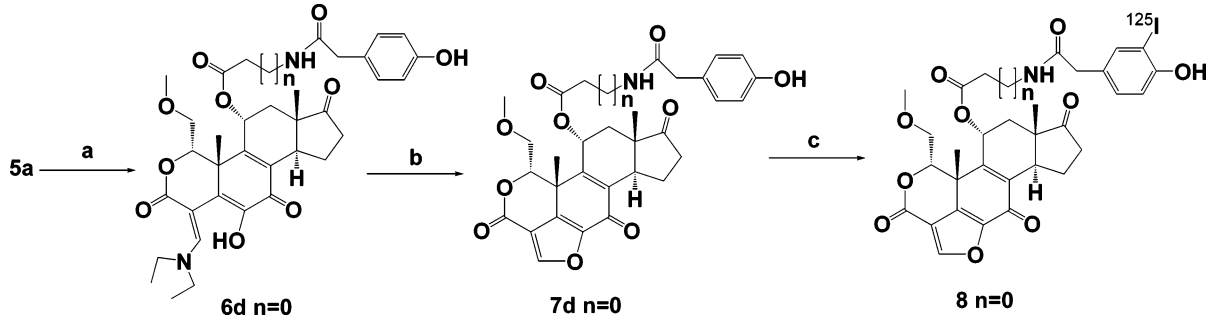
Synthesis of C11 4-Hydroxy-3-iodophenylated Wm (7b). Compound **5a** was prepared as above. *For step a, Scheme 3*, EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride) (3.8 mg, 0.02 mmol) and 4-hydroxy-3-iodophenyl acetic acid (5.4 mg, 0.02 mmol) were dissolved in anhydrous dichloromethane (0.5 mL) and stirred for 10 min. To this solution was added **5a** (0.01 mmol based on **4a**), prepared in anhydrous dichloromethane (0.5 mL). The mixture was stirred at room temperature for 20 min. *Step b, Scheme 3*, furan ring closure, was performed as above. The final product was purified with RP-HPLC by *system 2*. An off-white solid was obtained by precipitation with hexane from a solution of dichloromethane. (1.6 mg, 22.7% for **7b** overall, 0.01 mmol **4a** based). Selected data for **7b**: HR-MS: $C_{31}H_{30}INO_{10}{\cdot}NH_4^+$, calcd 721.1258, found 721.1262. ^{1}H NMR (CDCl3, 400 MHz): 0.92 (3H, s, C*H*$_3$-13), 1.41−1.47 (1H, m, *H-12*), 1.72 (3H, s, C*H*$_3$-10), 1.97−2.08 (1H, m, *H-16*), 2.23−2.33 (1H, m, *H-15*), 2.55−2.62 (2H, m, *H-12, H-16*), 2.79−2.83 (1H, m, *H-14*), 2.87−2.91 (1H, m, OCH_2), 3.15−3.22 (1H, m, *H-15*), 3.27 (3H, s, CH_3O), 3.29−3.58 (3H, m, OCH_2, ArCH_2CO), 3.85 (1H, dd, J_1 = 5.6 Hz, J_2 = 18 Hz, NHCH_2CO), 4.18(1H, dd, J_1 = 5.6 Hz, J_2 = 18 Hz, NHCH_2CO), 4.73 (1H, d, J = 6.4 Hz, *H-1*), 5.64 (1H, broad, O*H*), 6.15 (1H, td, J_1 = 8 Hz, J_2 = 2.4 Hz, *H-11*), 6.32 (1H, t, J = 5.6 Hz, N*H*), 6.91 (1H, d, J = 8.4 Hz, Ar*H*), 7.17 (1H, dd, J_1 = 3 Hz, J_2 = 8.4 Hz Ar*H*), 7.54 (1H, d, J = 3 Hz, ArH) and 8.27 (1H, s, *H-21*). ^{13}C NMR: ($CDCl_3$, 100 MHz): 14.7, 23.1, 26.6, 35.9, 36.3, 40.5, 41.0, 42.0, 44.2, 49.3, 60.2, 71.3, 73.3, 77.4, 86.0,

Scheme 4. Synthesis of C11 Fluorescein Wm (7c)[a]



[a] Reagents and conditions: (a) **4b**, diethylamine, dichloromethane, room temperature, 6 h (35.3%). (b) **5b**, fluorescein 5(6)-isothiocyanate, DMF, DIPEA, room temperature, overnight; (c) 1 N HCl, 1,4-dioxane, room temperature, 24 h, (8.9%, overall, 0.02 mmol **4b** based).

Scheme 5. Synthesis of ^{125}I Labeled C11 4-Hydroxy-3-iodophenylated Wm (8)[a]



[a] Reagents and conditions: (a) **5a**, EDC, 4-hydroxyphenylacetic acid, room temperature, 20 min; (b) 1,4-dioxane, 1 N HCl, 24 h (17.3%, overall, 0.01 mmol **4a** based); (c) **7d**, NaI-125, iodogen, phosphate buffer (0.5 M, pH 7.4), room temperature, 1−2 min, (more than 85% based on NaI-125).

89.4, 114.3, 115.3, 115.7, 128.6, 130.9, 131.4, 138.5, 139.1, 141.2, 142.8, 145.0, 149.0, 150.4, 154.7, 157.6, 168.8, 171.4, 172.8, and 216.0 (rotomer could be observed).

Synthesis of C11 Fluorescein Wm (7c). The reaction of **5a** with FITC and NHS esters of fluorescein was unsuccessful, presumably on steric grounds, so **5b** with an 11-carbon spacer between the primary amine and Wm was synthesized for fluorescein attachment (Scheme 4). *Fmoc Deprotection of 4b*. To **4b** (9.6 mg, 0.01 mmol) in anhydrous dichloromethane (1 mL) was added diethylamine (0.2 mL). After stirring for 6 h at room temperature, the solvent was evaporated under reduced pressure. The residue was fractioned by preparative silica gel TLC plate (dichloromethane:MeOH:triethylamine = 100:10:1). A yellow foam was obtained after the purification which contains trace amount of impurities generated during the concentration (2.3 mg, 35.3%). Compound **5b** was characterized by HR-MS: $C_{36}H_{54}N_2O_8$, calcd 643.3958 ($M + H^+$), found 643.3943.

For step b, Scheme 4, **5b** (0.02 mmol based on **4b**) and fluorescein 5(6)-isothiocyanate (15.6 mg, 0.04 mmol) were dissolved in DMF (1 mL) with diisopropylethylamine (0.05 mL) and stirred at room temperature for overnight. The closure of the furan ring (step c) was as above, with a final purification by HPLC with System 3. A brown solid was obtained (1.7 mg, 8.9% overall yield, based on 0.02 mmol **4b**). The proton NMR of **7c** lacked some structural detail due to the mixture of 6(5)-isomers in the starting FITC. However, key structural information was obtained confirming the structure **7c**: (1) a singlet at 11.94 ppm indicated the hydroxyl proton on the carboxylic acid fragment of fluorescein; (2) a singlet at 8.27 ppm indicated the furan ring was closed as in Wm; (3) a broad peak at 6.18 ppm indicated that C-11 hydroxy group was acylated; (4) strong peaks at the higher field overlapped with each other, indicating the existence of the 11-amino-undecanoyl ester linkage between Wm and fluorescein. The HR-MS supported the structure **7c**: $C_{53}H_{54}N_2O_{13}S$: calcd 959.3427 ($M + H^+$), found, 959.3434. UV absorbance (λ_{max} = 449.79 nm in methanol) and fluorescence spectra (λ_{ex} = 450 nm, λ_{em} = 515.9 nm in methanol) further indicated the presence fluorescein.

Synthesis of ^{125}I Labeled C11 4-Hydroxy-3-iodophenylated Wm (8). Compound **5a** was converted to **7d** with the similar procedures as **7b** without the isolation of **6d** except the application of 4-hydroxyphenyl acetic acid as one of the reactants (Scheme 5). **7d** was purified by reverse-phase HPLC (system 2) and identified by NMR and MS techniques**.** Iodination of the **7d** was accomplished by using iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) as the oxidant in the precoated tubes for 1−2 min. A stock solution of iodogen in dichloromethane with 0.5 μg/μL concentration (10 μL) was transferred to a V-shape vial. An iodogen precoated tube was made by blowing the above vial to dry with N_2 flow. Then phosphate buffer (0.5 M, pH 7.0) (10 μL) was added to the vial. A solution of 125-NaI (1 μL, 400−500 μCi) and a solution of **7d** in DMSO (1 μg/μL) (1 μL) were added sequentially. The mixture was vortexed for 1−2 min.

PI3 Kinase Assay. The assay was that of Cantley and co-workers with minor modifications (*21*). Briefly, CHO-K1 cells were lysed with lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 20 mM NaF, 10 mM NaPPi, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 4 μg/mL each of leupeptin, aproteinin, and pepstatin). The lysate was

clarified by centrifugation, and PI3K was immunoprecipitated overnight at 4 °C with a rabbit polyclonal antiserum against the p85 regulatory subunit of PI3K. The immunoprecipitate was captured on protein-A Sepharose beads and washed thrice with IP wash buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 20 mM NaF, 10 mM NaPPi, 50 mM β-glycerophosphate) and thrice with kinase assay buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5 mM EGTA). The immunoprecipitated PI3K was preincubated with a lipid substrate mix of phosphatidylserine, phosphatidylinositol, pnosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (in HEPES pH 7.4 30 mM, EGTA 1 mM) at room temperature for 10 min with or without the presence of Wm. Kinase reaction was initiated with the addition of γ-[32]P-ATP (in HEPES pH 7.4 30 mM, $MgCl_2$ 10 mM and ATP 0.5 mM) and allowed to proceed for 10 min at room temperature before termination with 4 M HCl. The phospholipid products were extracted with MeOH:$CHCl_3$ (1:1). The organic fraction contained the phospholipids that were separated by TLC with 2 M acetic acid:*n*-propanol (35:65) as solvent. Phospholipid signal was quantified using a phosphoimager screen. The percent inhibition of enzyme activity as a function of Wm concentration was analyzed to yield IC_{50}s using a four-parameter fit on GraphPad Prism software.

Reaction of C11-Modified Wm's with Proteins. ^{125}I labeled C11 4-hydroxy-3-iodophenylated Wm (**8**) was reacted with PI3K purified by immunoprecipitation as above. Briefly, 300 μg lysate was incubated with 1000 nM **8** (250 μCi) for 30 min at 4 °C. Proteins were then solubilized in reducing SDS sample buffer and resolved by SDS−PAGE. Gels were dried and visualized by exposure to a phosphorimager cassette for 18 h. For the reaction of C11 biotinylated Wm with BSA, 10 mg/mL in PBS was reacted with 10 or 100 μM of either C11 biotin Wm or as a positive control the *N*-hydroxysuccinimide ester of biotin (Pierce Chemical, Rockford IL) for 1 h at 37 °C. Two micrograms of protein was then subjected to SDS−PAGE followed by reaction with avidin-horseradish peroxidase (Pierce Chemical) diluted 1/20 000 in PBS containing 0.05% Tween 20 (2 h, RT, shaking). Visualization was with 3,3′-diaminobenzidine and 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, MO) at 20 mg of each, in 100 mL of PBS with 20 μL of 30% hydrogen peroxide.

RESULTS

The synthetic strategy of C11 reporter Wm's has several key features. Wm was reacted in a polar solvent methanol with diethylamine to form a C20-modified and C-11-desacetylated adduct (**2**) which was converted to the furan ring-closed derivative **3** according to the method of Creemer et al. (*20*). Compound **3** was reacted with *N*-Fmoc-glycyl acid chloride or *N*-Fmoc-11-aminododecanoyl acid chloride to yield **4a** and **4b**, respectively, with decent yields, quality, and stability. These compounds were stable during storage at −20 °C for at least 3 months and were obtained in high yield (50−70%). An instability was noted when the storage of **5a** and **5b** was attempted. Due to the base sensitivity of the compound, Fmoc deprotection was accomplished with milder base diethylamine in nonpolar solvent dichloromethane, which removed the Fmoc group and opened the furan ring in a useful manner. Reaction of FITC with **4a** was unsuccessful, presumably on steric grounds, so **4b** was synthesized with an 11-carbon spacer between the primary amine and Wm structures. The furan ring reformed readily in acidic conditions.

Table 1. Inhibition of PI3 Kinase by C11 Reporter Wm's

compound	IC_{50} (nM)[a]
Wm (**1**)	1.63 (1.52−1.75)
C11 biotinylated Wm (**7a**)	6.11 (5.31−7.03)
C11 4-hydroxy-3-iodophenylated Wm (**7b**)	11.02 (9.95−12.2)
C11 fluoresceinated Wm (**7c**)	64.9 (54.2−77.8)

[a] 95% confidence intervals within parentheses.

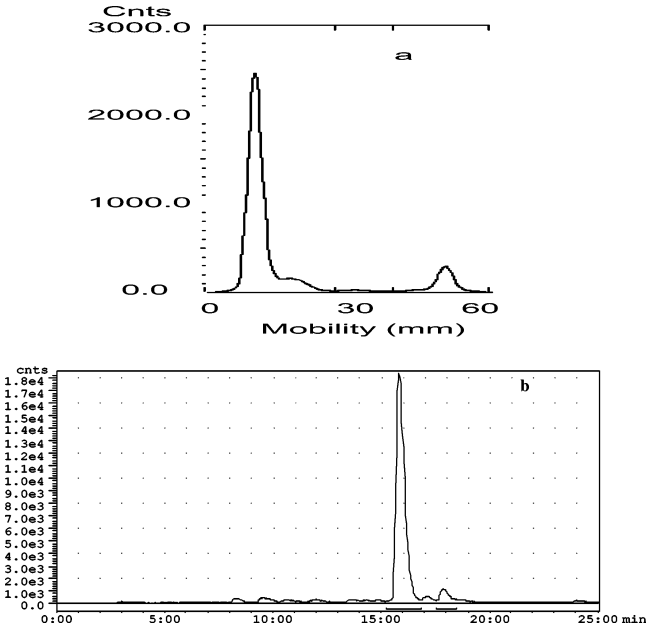


Figure 2. Iodination of C11−4-hydroxyphenylated Wm (**7d**). (a) TLC scan after iodination of **7d**. A reverse phase-TLC with C18-modified silica gel was developed with a mixture of PBS and methanol (2:1). (b) HPLC of the iodination of **7d** with γ-ray detector, C18-modified silica gel column (4.6 × 250 mm), and gradient was from water:acetonitrile 80:20 to 15:85 in 25 min. The peak of radioactivity at 15.48 min corresponded to the peak of C11 4-hydroxy-3-iodophenylate Wm (**7b**) as the internal standard with UV detector at 15.50 min.

The three C11 reporter Wm's were then analyzed for their ability to inhibit PI3 kinase as summarized in Table 1. An IC_{50} of 1.63 nM for Wm (**1**) was obtained, in good agreement with the IC_{50} of 1.8 nM reported earlier (*20*). The C11 biotinylated Wm (**7a**) and C11 4-hydroxy-3-iodophenyl Wm (**7b**) had IC_{50}s of 6.11 and 11.02 nM, respectively, modestly higher than Wm, while the C11 FITC Wm (**7c**) had an IC_{50} to 64.9 nM.

We used a C11 4-hydroxyphenyl Wm (**7d**) as substrate for iodination with 125-NaI by the iodogen method as shown in Scheme 5. About 85−100% of iodine reacted with **7d** as shown by the larger peak of low mobility in Figure 2a. The smaller peak of high mobility was identified as radioactive iodide. The iodinated mixture was further characterized by HPLC using a radioactive detector as shown in Figure 2b. The major peak at 15.48 min had been verified with the retention time consistency with **7b** at 15.50 min by using **7b** as an internal standard with a UV detector.

We next examined whether our C11 ^{125}I labeled 4-hydroxy-3-iodophenyl Wm (**8**) could be detected after reaction with proteins, and whether it could indicate the molecular weight of the protein with which it reacted. C11 ^{125}I labeled 4-hydroxy-3-iodophenyl Wm (**8**) was reacted with a p85 immunoprecipitate from CHO cells as shown in Figure 3. After reaction, radioactivity was

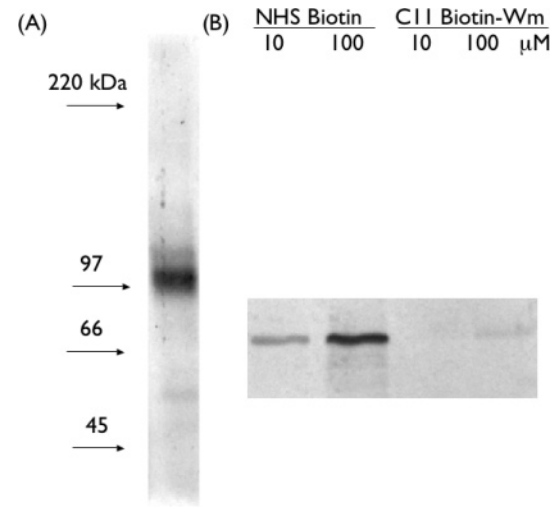


Figure 3. Visualization of the reaction of C11-modified Wm's with proteins. (A) The C11 ^{125}I 3-iodo-4-hydroxyphenyl Wm (**8**) was reacted with purified PI3K, subjected to SDS−PAGE and visualized by autoradiography. Molecular markers were myosin, phosphorylase, bovine serum albumin, and ovalbumin. (B) The C11 biotinylated Wm (**7a**) or the *N*-hydroxysuccinimide ester of biotin at 10 or 100 μM was reacted with purified bovine serum albumin at 10 mg/mL (2 h, 37 °C), subjected to SDS−PAGE and visualized with an avidin-horseradish peroxidase and substrate.

detectable and was associated with a band of about 100 kDa, which is a mobility similar to that expected for p110 catalytic subunit of PI3K (Figure 3A). We also examined the ability of our C11 biotinylated Wm (**7a**) to be detected after exposure to a non-PI3K protein, bovine serum albumin (BSA), under conditions of high protein (20 mg/mL) which would favor this reaction. As a positive control, BSA was reacted with the *N*-hydroxysuccinimide (NHS) ester of biotin. As shown in Figure 3B, only a trace amount of biotinylated BSA was seen with 100 μM C11 biotinylated Wm. Thus both C11 ^{125}I labeled 4-hydroxy-3-iodophenyl Wm (**8**) and the C11 biotinylated Wm (**7a**) are Wm probes that can be used to monitor the reactivity of Wm with proteins.

DISCUSSION

The inhibition of PI3 kinase with C11-modified Wm's can be evaluated in light of previous studies of Wm-based PI3 kinase inhibitors. Creemer et al. found that the replacement of the C11 acetyl group (CH_3COO) with a variety of modestly larger functional groups yield compounds with lower IC_{50}s than Wm (**1**). For example replacement of the C11 acetyl with a propyl group (CH_3-CH_2COO) yielded an IC_{50} of 0.38 nM, while removal of the acetyl group (11-*O*-desacetylwortmannin) had an IC_{50} of 10 nM (Wm IC_{50} = 1.8 nM). These results are supported by the crystal structure of the Wm binding in the ATP site of PI3 kinase, indicating hydrophobic amino acid side chains in the vicinity of the C11 interact favorably with small hydrophobic groups and unfavorably with the hydrophilic hydroxyl group of 11-*O*-desacetylwortmannin (*16*). The modest increase in IC_{50}s we obtained for the C11 biotinylated (**7a**) and 4-hydroxy-3-iodophenyl Wm's (**7b**), 3.7 and 6.8-fold respectively, suggest these compounds slightly exceed the constraints of the PI3 kinase Wm binding site for C11 modifications. The larger C11-fluorescein-modified Wm (**7c**) we synthesized appears to exceed the constraints of the C11 pocket, evident by a 39.8-fold increase in IC_{50}. Thus PI3 kinase binds C11-modified Wm's, provided the modification remains about the size of a biotin moiety or smaller and suggest that future C11 Wm probes must utilize small and hydrophobic modifications at C11, to maximize their interaction with ATP site of PI3 kinase.

A goal of this work was to determine whether various reporter Wm's might inhibit PI3 kinase sufficiently to permit their use with in vivo systems. It would be reasonable to evaluate these probes in vivo, in light of the affinities and losses in affinity often associated with the synthesis of radioactive steroids (*22*−*24*). In addition, our results suggest that the design of further fluorescent C11 Wm's that have a high affinity for PI3 kinase might be possible, but that the size of the modification and fluorochrome should be kept as small as possible.

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