# Wortmannin-C20 Conjugates Generate Wortmannin

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We report on C20-6-(N-methylamino)hexanoic conjugates of wortmannin featuring a tertiary enamine attached to the C20 that inhibit phosphoinositol-3-OH kinase (PI3K) by producing wortmannin (Wm) through an intramolecular attack. The generation of Wm by these conjugates permits the design of Wm based PI3K inhibitors that need not fit into the ATP pocket of PI3K, including Wm conjugates of BSA, IgG, or beads. Wm generating WmC20-N(Me)-hexanoate conjugates offer an approach to the design of targeted or slow release forms of Wm which may inhibit PI3K in tissues more selectively than the parent Wm, a compound which has desirable anti-inflammatory and anti-proliferative activities but which also has a variety of toxic effects.

## Introduction

Wortmannin (Wm) is a steroid-like molecule that reacts covalently with a lysine in the ATP binding site of the catalytic subunit of phosphoinositol-3-OH kinase (PI3K).[1,2] The central role played by PI3K in cell proliferation and immune regulation suggests that PI3K inhibitors might be developed as anti-cancer or anti-inflammtory drugs.[3−6] Wm and its derivatives have long been investigated as anti-inflammatory agents (1970s−present)[5,7−11] and more recently for their anti-cancer activities (mid-1990s−present).[12−15] Wm is growth inhibitory for tumor cell lines from the pancreas,[16] lung,[17] and breast.[15,18] The activity of PI3K, inclusive of all subtypes, is essential for normal function in diverse areas of biology including platelet adhesion,[19,20] insulin action,[21,22] growth factor activity (PDGF, VEGF Her2/neu, EGF),[23] cell proliferation,[4,23,24] and immune function.[25−27] Wm is also used to inhibit PI3K and demonstrate the role of the enzyme, and pathways it controls, in biological phenomena.

The use of Wm as a pharmaceutical is hampered by its toxicity which was described as hemorrhagic in the early literature,[28−30] perhaps because of its ability to inhibit PI3K in platelets.[20] More recent studies have focused on Wm's hepatic and myelosuppressive toxicities.[31,32] A second problem limiting the use of Wm as a pharmaceutical is its instability.[33] Thus, despite considerable literature suggesting that Wm or Wm derivatives have desirable therapeutic effects, development of Wm based drugs has been hindered by their considerable toxicity and instability. Attempts to overcome the drawbacks of Wm have including synthesis of Wm derivatives at the C11 position and C17 position.[34,35] The chemistry and biology of Wm have recently been reviewed.[36]

One class of efforts to develop Wm derivatives as PI3K based pharmaceuticals has focused on the modification of Wm at the C20 position by amines or thiol compounds.[31,32] PX-866, formed by reaction of Wm with diallylamine at C20, has an $IC_{50}$ for PI3K 12 times lower than the parent Wm and has shown promise in animal tumor models. The superiority of WmC20 derivatives over Wm as PI3K inhibitors may be due to an improved fit for the C20 derivatives into the ATP site or because the C20 substituents serve as superior leaving groups, enhancing the rate of attack by the lysine residue and covalent modification of the enzyme[32] as shown in Figure 1 (dotted arrow). However, a second mechanism proposed here postulates that WmC20 derivatives generate Wm, which then binds PI3K (Figure 1, solid arrow). Even though both mechanisms require detachment of the substituents at C20, and covalent modification of Wm by an amino group in the ATP site, the difference between them has significant implications. The ability to generate Wm, or lack thereof, might explain the relative potency of Wm based compounds. In addition, the self-generation mechanism permits the design of Wm based compounds as PI3K inhibitors that do not need to fit into the ATP pocket of PI3K.

Our goals were (i) to demonstrate that a WmC20 derivative could self-activate or generate Wm and (ii) to demonstrate that WmC20 derivative maintained the property of self-activation after conjugation to the amino groups of proteins or solid phases. To accomplish these goals, we synthesized a C(20) 6-(N-methylamino)-hexanoic derivative of Wm (**2a**), featuring a tertiary enamine attached to the C20 (Figure 2). We also synthesized a C(20) 6-amino-hexanoic derivative of Wm (**2b**), featuring a secondary enamine attached to C20. Compound **2b**, a Wm derivative referred to as WmC20-NH-hexanoic acid, is shown with the six membered hydrogen bond structure as proposed by Norman,[13] which we confirmed by the presence of a proton peak at 9.88 ppm (hydrogen bonded NH proton) and by a shift in the position of other protons (Figure 6B). We find that the self-activation of compound **2a**, referred to as WmC20-N(Me)-hexanoic acid, was maintained after conjugation to proteins or solid phases. Our results suggest that self-activating Wm conjugates might be designed to produce locally high concentrations of Wm in vivo and offer a new approach to the selective inhibition of PI3K.

## Materials and Methods

**General Description.** Wortmannin was a gift of the natural products branch of the NCI. All reagents and solvents (Aldrich) were of standard quality. Silica gel (open column chromatography) was 60−20 mesh (J. T. Baker). BSA and mouse IgG were from Sigma. Dynal M-270 amine magnetic beads were purchased from Dynal Biotech. Pre-packed PD-10 columns were from Amersham Biosciences. NMR was performed on Varian 400 MHz instrument

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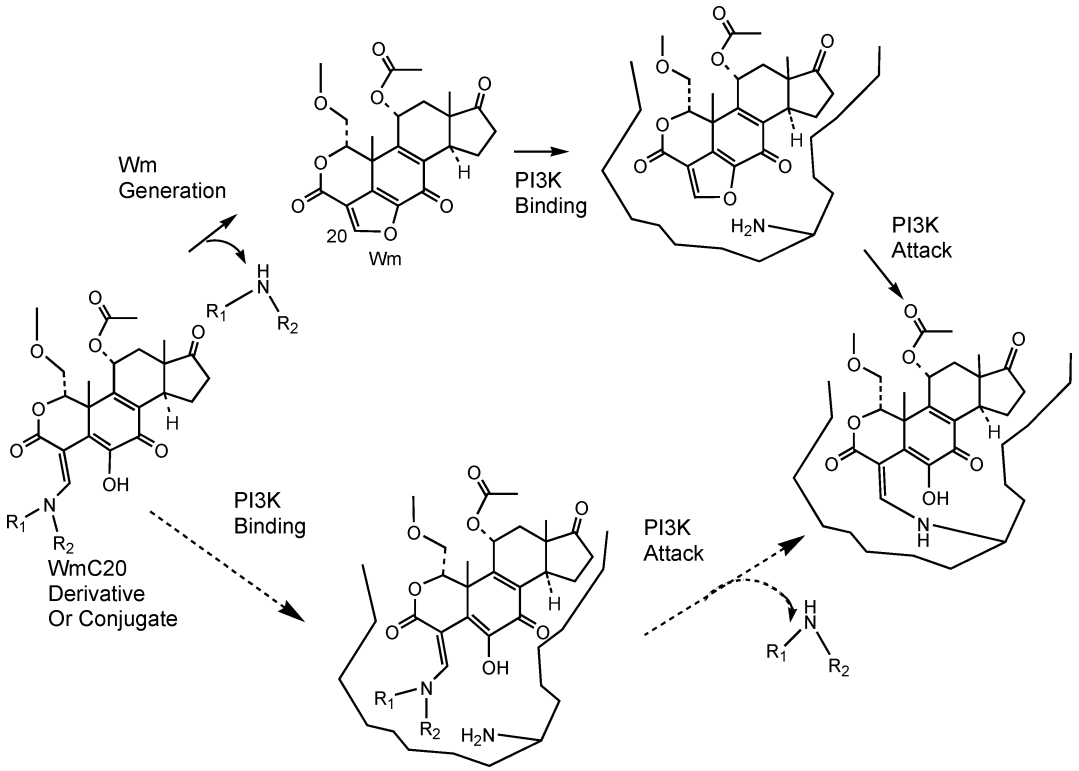


**Figure 1.** Possible mechanisms of action of Wm C20 Derivatives**.** Wm binds to PI3K, with attack by lysine 833 of PI3K. For the general mechanism of Wm action, see refs 1 and 2. We propose that Wm C20 derivatives generate Wm through an intermolecular attack, which then inhibits PI3K (solid arrows). Wm C20 derivatives could also bind to PI3K with attack by lysine as proposed (dotted arrows).[31,32]

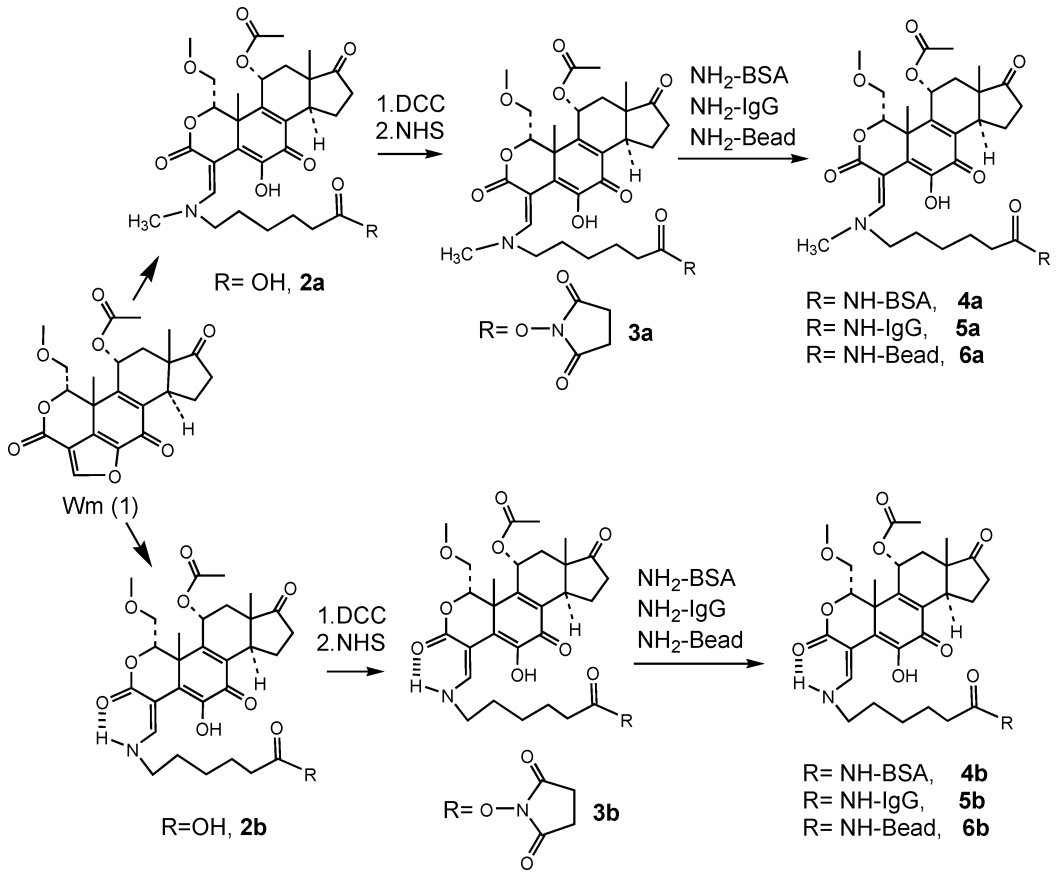


**Figure 2.** Synthesis of WmC20 derivatives. Wm was modified by reaction with 6-N(Me)-hexanoic acid or 6-NH-hexanoic acid followed by formation of the N-hydroxysuccinimide esters **3a** or **3b**. Compounds **3a** or **3b** are then attached to the amine groups of BSA, IgG, or amine functionalized beads.

with $CDCl_3$ as solvent, while mass spectra were obtained on a Micromass LCT instrument with time-of-flight ESI technique. HPLC (Varian Prostar 210 with a variable wavelength PDA 330 detector) employed reverse phase C18 columns (VYDAC, Cat.#:

218TP1022 for synthesis; Varian, Cat. #: R0086200C5 for analysis) with water (Millipore, containing 0.1% trifloroacetic acid) (buffer A) and acetonitrile (containing 20% buffer A) (buffer B) as elution buffer. *System 1:* buffer A/buffer B (80:20) isocratic for 5 min, linear gradient to buffer A/buffer B (20:80) over 40 min, then the gradient back to 80:20 in 5 min and isocratic for 5 min. Flow: 4.9 mL/min. $\lambda_{max}$: 418 nm (used for purification of **2a**). *System 2:* buffer A/buffer B (80:20) isocratic for 5 min, linear gradient to buffer A/buffer B (20:80) over 25 min and then isocratic for 5 min, then the gradient back to 80:20 in 5 min and isocratic for 5 min. Flow: 6.0 mL/ min. $\lambda_{max}$: 408 nm (used for purification of **2b**). *System 3*: buffer A/buffer B (70:30) linear gradient to buffer A/buffer B (20:80) over 15 min, then gradient back to 70:30 in 3 min and isocratic for another 5 min. Flow: 1.0 mL/min. $\lambda_{max}$: 258 nm (Figure 3A,C). *System 4*: buffer A/buffer B (99:1) linear gradient to buffer A/buffer B (40:60) over 46 min, then gradient back to 90:1 in 5 min and isocratic for another 5 min. Flow: 1.0 mL/min. $\lambda_{max}$: 250 nm (Figure 3B,D).

**Synthesis of Wm C20 Derivatives and Conjugates.** Relationships between the compounds synthesized are shown in Figure 2. 6-(N-Methylamino)-hexanoic acid was made according to literature.[37]

**Cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione,5-(acetyloxy)-1-{[N-(5-carboxylpentyl)-N-methylamino]methylene}-4,4a,5,6,6a,8, 9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-, (4S,4aR,5R,6aS,9aR)- (9CI) (Referred To as WmC20-N(Me)-hexanoic Acid 2a).** Wortmannin (42.8 mg, 0.1 mmol), 6-(N-methylamino)hexanoic acid hydrogen chloride (90 mg, 0.5 mmol), and triethylamine (20 $\mu$L) were mixed in anhydrous DMSO (1 mL). The mixture was stirred at room temperature until complete as monitored by TLC (about 1.5 h). After dilution with 50% acetonitrile in water (1:1) before the injection, the mixture was purified by HPLC (system 1) and gave a yellow powder after lyophilization. Analysis of **2a** by HPLC (system 3) showed less than 3% of Wm: 39.5 mg, 68.9%. MS: $C_{30}H_{39}NO_{10}$, calcd 574.2652 ($M + H^+$), found 574.2648. $^1$H NMR ($CDCl_3$): 0.85 (3H, s, C18$-CH_3$), 1.41−1.54 (2H, m, $CH_2CH_2CH_2-$), 1.58 (3H, s, C19$-CH_3$), 1.65−1.78 (4H, m, $-CH_2CH_2CH_2-$), 1.80−1.85 (1H, dd, $J_1 = 14.8$ Hz, $J_2 = 4.63$ Hz, H-12), 2.06 (3H, s, $CH_3CO$), 2.09−2.20 (1H, m, H-16), 2.22−2.33 (1H, m, H-15), 2.37−2.43 (3H, m, H-16, $CH_2CH_2COOH$), 2.54−2.61 (1H, m, H-12), 2.79 (3H, broad, $NCH_3$), 2.91−3.03 (2H, m, H-14, $CH_2OCH_3$), 3.12−3.24 (2H, m, H-15, $CH_2OCH_3$), 3.25 (3H, s, $CH_3O$), 3.39−3.56 (2H, m, broad, $NHCH_2$), 4.05 (2H, broad, OH), 4.49 (1H, dd, $J_1 = 2$ Hz, $J_2 = 7.6$ Hz, H-1), 6.06 (1H, dd, $J_1 = 4.8$ Hz, $J_2 = 7.2$ Hz, H-11), 8.18 (1H, s, H-20). The $^{13}$C NMR could not be obtained due to Wm generation in the spectra recording.

**Cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione,5-(acetyloxy)-1-{[N-(5-carboxylpentyl)amino]methylene}-4,4a,5,6,6a,8,9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-, (4S,-4aR,5R,6aS,9aR)- (9CI) (Referred To as WmC20-NH-hexanoic Acid 2b).** Wm (42.8 mg, 0.1 mmol), 6-aminohexanoic acid (65.5 mg, 0.5 mmol), and triethylamine (20 $\mu$L) were mixed in anhydrous DMSO (1 mL). The reaction was monitored as above, purified by HPLC (system 2), and after lyophilization a yellow powder was obtained. HPLC analysis showed a single peak to prove its high purity: 42.5 mg, 76.0%. MS: $C_{29}H_{37}NO_{10}$, calcd 560.2495 ($M + H^+$), found 560.2499. NMR ($CDCl_3$, ppm): $^1$H NMR ($CDCl_3$): 0.83 (3H, s, C18$-CH_3$), 1.43−1.51 (2H, m, $-CH_2CH_2CH_2-$), 1.53 (3H, s, C19$-CH_3$), 1.68−1.75 (4H, m, $-CH_2CH_2CH_2-$), 1.86−1.90 (1H, dd, $J_1 = 14.8$ Hz, $J_2 = 2.8$ Hz, H-12), 2.05 (3H, s, $CH_3CO$), 2.24−2.37 (3H, m, H-15, H-16), 2.42 (2H, t, J = 7.4 Hz, $CH_2$-$CH_2COOH$), 2.54−2.63 (1H, m, H-12), 2.84−2.88 (1H, m, $CH_3$-$OCH_2$), 2.94−2.99 (1H, m, H-14), 3.14−3.26 (2H, m, H-15, $CH_3OCH_2$), 3.27 (3H, s, $CH_3OCH_2$), 3.42 (2H, q, $J_1 = 13.0$ Hz, $J_2 = 6.5$ Hz), 4.33−4.35 (1H, m, H-1), 4.84 (2H, broad, OH), 5.98−6.01 (1H, m, H-11), 8.55 (1H, d, J = 13.9 Hz, H-20), 9.81−9.92 (1H, m, NH). $^{13}$C NMR: 16.84, 21.14, 22.59, 24.29, 26.05, 26.43, 30.45, 33.62, 36.71, 38.59, 42.36, 42.41, 50.04, 59.42, 67.29, 73.33, 81.37, 88.43, 129.06, 137.09, 137.57, 151.13, 159.63, 166.40, 170.27, 178.01, 178.69, 218.29.

**NHS Ester, Cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione, 5-(acetyloxy)-1-{[N-(5-carboxylpentyl)-N-methylamino]-methylene}-4,4a,5,6,6a,8, 9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-, (4S,4aR,5R,6aS,9aR)- (9CI) (3a).** Compound **2a** (15.8 mg, 0.0276 mmol) was mixed with N-hydroxysuccinimide (38 mg, 0.33 mmol) and dicyclohexylcarboimide (68.2 mg, 0.33 mmol) in anhydrous acetonitrile (1 mL). The mixture was stirred at room temperature for 30 min. The solid residue was obtained by filtration and washed with anhydrous acetonitrile, and the solvent was removed by partial vacuum. The residue was fractioned by silica gel chromatography with anhydrous ethyl acetate:hexane (5:1−10:1). A light yellow solid was obtained by precipitation with anhydrous hexane from trace amount of anhydrous ethyl acetate: 17 mg, 92%. MS: $C_{34}H_{42}N_2O_{12}$, calcd 671.2816 ($M + H^+$), found 671.2819.

**NHS Ester, Cyclopenta[5,6]naphtho[1,2-c]pyran-2,7,10(1H)-trione, 5-(acetyloxy)-1-{[N-(5-carboxylpentyl)amino]methylene}-4,4a,5,6,6a,8,9,9a-octahydro-11-hydroxy-4-(methoxymethyl)-4a,6a-dimethyl-, (4S,4aR,5R,6aS,9aR)- (9CI) (3b).** Compound **2b** (11.2 mg, 0.02 mmol) was mixed with N-hydroxysuccinimide (27.6 mg, 0.24 mmol) and dicyclohexylcarboimide (49.5 mg, 0.24 mmol) in anhydrous acetonitrile (1 mL) and handled as for **3a**. The residue was fractioned by silica gel chromatography with anhydrous ethyl acetate:hexane 3:1. A light yellow solid was obtained by precipitation with anhydrous hexane from trace amount of anhydrous ethyl acetate: 12.4 mg, 94.6%. MS: $C_{33}H_{40}N_2O_{12}$, calcd 657.2659 ($M + H^+$), found 657.2669.

**Conjugation of the WmC20 Derivatives 2a or 2b to Proteins or Beads.** Proteins were dissolved in PBS, pH 6.8 at 4−10 mg/mL. The WmC20 NHS esters (**3a** or **3b**) were dissolved in DMSO at 40 mg/mL (60 mM), and 10−30 $\mu$L, depending on the loading ratio, was added to 0.5 mL of protein. Reaction was for 1−1.5 h at 37 °C. Unreacted Wm derivative was removed with PD-10 columns. Concentrations of proteins were determined spectrophotometrically, subtracting the OD280 from the Wm derivative before determining protein. The concentration of Wm was determined from its absorbance at 418 nm using an extinction coefficient of 7058 $cm^{-1}$ $M^{-1}$ we determined. Wm to protein molar ratios were as follows: **4a** (**2a**/BSA) = 4.9; **4b** (**2b**/BSA) = 8.7; **5a** (**2a**/IgG) = 23; **5b** (**2b**/IgG) = 31. Beads (Dynal M-270, amine functionalized) in 0.6 mM PBS ($2 \times 10^9$ beads/mL) were washed using a Dynal magnetic separator and suspended in 0.5 mL of DMSO/PBS (50:50 v/v). WmC20 NHS ester **3a** or **3b** (50 $\mu$L) dissolved in DMSO at 40 mg/mL (60 mM) was added to beads. After 1 h at 37 °C, unreacted Wm derivatives were removed by magnetic separation of the beads.

**Generation of Wm with WmC20 Derivatives and WmC20 Conjugates.** Solutions of **2a** and **2b** (200 $\mu$L of about 1.5 mM in Wm or Wm derivatives) were made by adding 30 mM WmC20 derivative in DMSO to PBS buffer (pH 6.8). All experiments were in PBS, pH 6.8. Solutions were incubated (37 °C for the indicated time) and analyzed by applying 10 $\mu$L plus 2 $\mu$L of internal standard (1 $\mu$g of methyl-4-hyrdroxybenzoate, MHB) to the HPLC. System 3 was used for **2a** and Wm while system 4 was used for **2b**.

Compounds **4a** (1.8 mg) and **4b** (1.3 mg) were dissolved in 0.4 mL of PBS buffer, incubated at 37 °C for the indicated time, and applied to HPLC (system 3 for **4a**, system 4 for **4b**). Initial concentrations used when modeling Wm generation (eq 1, Figure 4A) were determined by weight for **2a** and **2b** and spectrophotometrically (extinction coefficients: **2a**, 7085 $cm^{-1}$ $M^{-1}$ at 418 nm; **2b**, 5505 $cm^{-1}$ $M^{-1}$ at 408 nm) for **4a**, **4b**, **5a,** and **5b**.

**Stability of Wm, WmC20 Derivatives, and WmC20 Conjugates.** To obtain the stability of Wm in the presence of BSA, Wm (0.45 mg, 1.04 $\mu$mol) and BSA (1.8 mg) were incubated in PBS buffer (400 $\mu$L) and acetonitrile (60 $\mu$L) at 37 °C for various times. MHB was added as above followed by HPLC (system 3). To determine the stability of Wm in the presence of N-acetyl lysine or proline, Wm (about 1.5 mM) in PBS with 10 mM of N-acetyl lysine or proline (pH 6.8, 400 $\mu$L) and acetonitrile (60ul) in a 1.5 mL tubes were incubated at 37 °C. At different times, a 10 $\mu$L

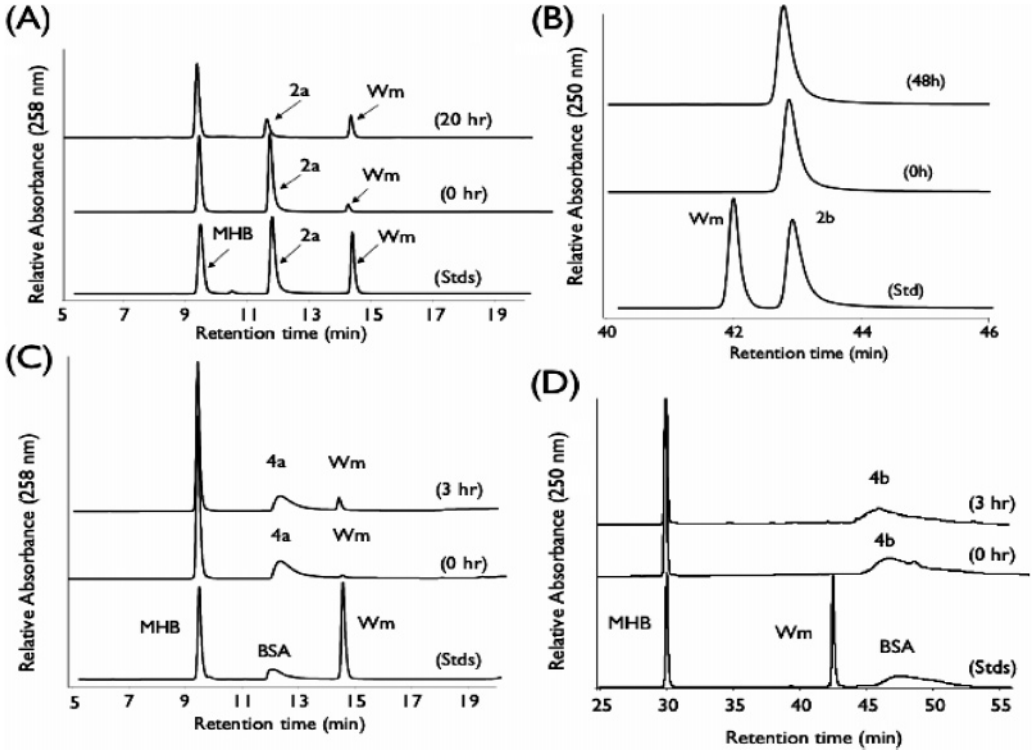


**Figure 3.** Formation of Wm from Wm C20 derivatives and conjugates. (A) Formation of Wm from **2a**. The internal standard is MHB (methyl-4-hydroxybezoate). (B) Lack of formation of Wm from **2b**. (C) Formation of wortmannin from **4a**. (D) Lack of formation of Wm from **4b**. HPLC system 3 was used for A and C while HPLC system 4 was used for B and D.

solution was mixed with 2 $\mu$L internal standard solution in DMSO (2 $\mu$g MHB) and analyzed by HPLC (system 3).

For both Wm generation and Wm stability studies, the internal standard (IS) ratio was calculated for each sample by dividing the peak area of the samples by the area of internal standard (MHB). To obtain the concentration of Wm generated (Figure 4A), standard curves were obtained by using 1−5 $\mu$g of the appropriate WmC20 derivatives or conjugates and IS ratio.

PI3K activity was measured as described by Cantley and co-workers,[38] with minor modifications.[39] The assay uses enzyme from CHO-K1 cells and assesses lipid phosphorylation by $^{32}$P labeled ATP after a 30 min incubation.

## Results

We hypothesized that a Wm derivative with a tertiary enamine at C20 would generate Wm, while a Wm derivative with a secondary enamine at C20 would not, based on the work of Norman discussed below. We therefore synthesized WmC20-N(Me)hexanoic acid (**2a**) and conjugated it to the amino groups of BSA, IgG, or amine functionalized beads to obtain the WmC20-N(Me)hexanoic acid conjugates, **4a**, **5a**, or **6a**. Similar reactions were performed with WmC20-NH-hexanoic acid (**2b**) to obtain **4b**, **5b**, and **6b**. This strategy allowed WmC20-NH-hexanoic acid based compounds to serve as negative controls for their WmC20-N(Me)-hexanoic counterparts in experiments where the generation of Wm was to be monitored.

We first examined the ability to generate Wm by diluting our Wm derivatives and conjugates into PBS (pH 6.8), the buffer used for all experiments, and analyzed the resulting samples for the presence of Wm by RP-HPLC. Figure 3A−D shows HPLC methods used to determine the formation of Wm in these experiments. With increasing incubation time, WmC20-N(Me)-hexanoic acid (**2a**) showed a progressive increase in Wm and decrease in **2a** (Figure 3A), which was not seen when a similar experiment was performed with **2b** (Figure 3B). Because **2b** and Wm had a difference in retention time of only about 1.0 min, the time scale was expanded on Figure 3B to clearly visualize the presence of Wm, which was not detected. It can be seen that **2a** generated Wm while **2b** did not, with a more complete time course for Wm generation from **2a** shown in Figure 4A. We next examined if the formation of Wm would occur if **2a** was conjugated to BSA to yield **4a**. After 3 h, **4a** generated Wm (Figure 3C), while Wm was not detected under the same conditions with **4b** (Figure 3D). The complete time course of Wm formation from **4a** is shown in Figure 4A. Figure 4A also shows the time course of Wm formation obtained when **2a** was conjugated to IgG (**5a**) or beads (**6a**). Thus WmC20-N(Me)-hexanoic acid (**2a**) and materials made with **2a** (**4a**, **5a**, and **6a**) generated Wm by our HPLC method. In contrast, Wm formation could not be detected with WmC20-NH-hexanoic acid (**2b**) or materials made from **2b** (**4b**, **5b**, **6b**).

A linear sequential model for the formation of Wm and subsequent decay was used to generate the lines shown in Figure 4B. The model yields eq 1 in the case where the Wm concentration as a function of time, $[\mathrm{Wm}]_t$, occurs from an initial concentration of **2a**, $[\mathbf{2a}]_i$.

$$[\mathrm{Wm}]_t = [\mathbf{2a}]_i\, k_1[\exp(-k_1 t) - \exp(-k_2 t)]/(k_2 - k_1) \quad (1)$$

A two parameter fit was employed to obtain $k_1$ and $k_2$ for **2a**, **4a**, and **5a** with data provided in Table 1. The amount of WmC20-N(Me)-hexanoic acid attached to beads (**6a**) could not be determined either by weight or spectrophotometrically, so a three parameter fit was employed to obtain values of $k_1$, $k_2$, and $[\mathbf{6a}]_i$. The simple linear formation and decomposition model (Figure 4B) satisfactorily described the time course of Wm formation with WmC20-N(Me)-hexanoic acid (**2a**) and conjugates made with it (**4a**, **5a**, and **6a**).

As these studies were being conducted, Holleran[33] reported that Wm had a half-life of 8−13 min in culture media at 37 °C, a value that seemed inconsistent with the kinetics of Wm

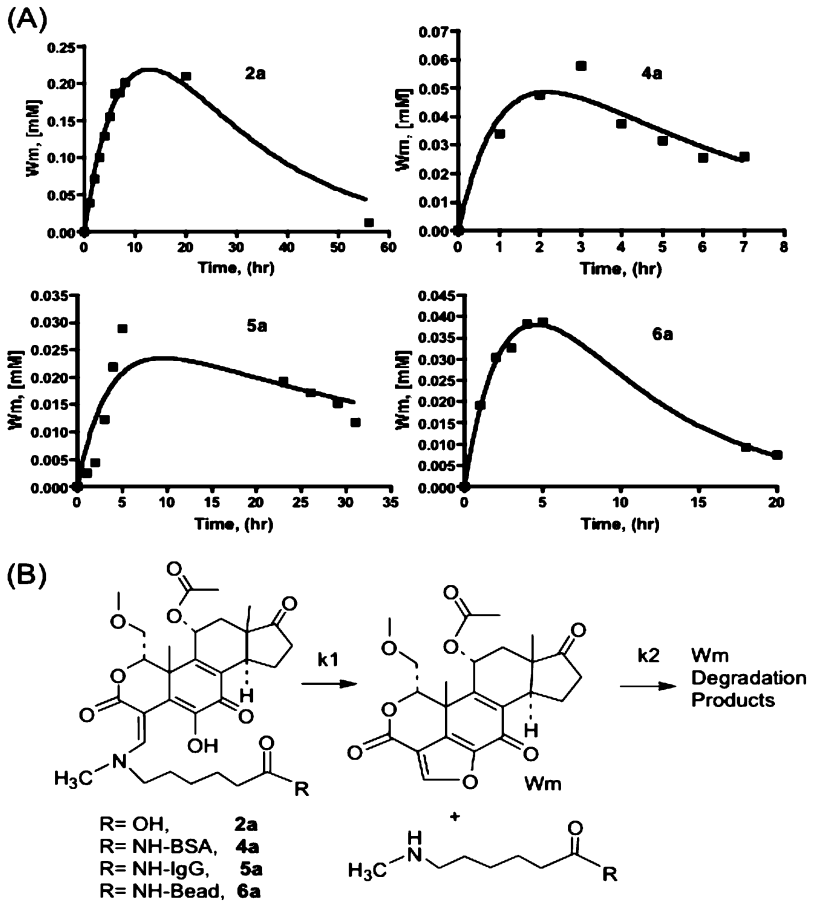


**Figure 4.** Time course of Wm generation from WmC20 N(Me)-hexanoic acid derivatives. (A) Time course of Wm generation and degradation by HPLC. Data were analyzed according to the model shown in B which yields eq 1. Values of $k_1$ and $k_2$ are given in Table 1.

**Table 1.** Constants for Wm Generation from Wm C20 Derivatives (Fit to Eq 1)[a]

| compd | initial concn of Wm derivative (mM Wm) | $k_1$ ($h^{-1}$) | $k_2$ ($h^{-1}$) |
|---|---|---|---|
| **2a** | 0.94 | 0.051 (0.045-0.056) | 0.114 (0.095-0.132) |
| **4a** (**2a**−BSA) | 0.35 | 0.190 (0.128-0.253) | 0.905 (0.707-1.103) |
| **5a** (**2a**−IgG) | 0.35 | 0.024 (0.013-0.034) | 0.282 (0.166-0.398) |
| **6a** (**2a**−bead) | 0.0750 (0.0414-0.109) | 0.308 (0.124-0.492) | 0.148 (0.076-0.220) |

[a] Concentrations of BSA and IgG were 0.065 mM and 0.011 mM, respectively. Concentration of beads was $3 \times 10^9$ beads/mL.

disappearance shown in Figure 5. To resolve this issue, we studied the disappearance of Wm in the presence of BSA, *N*-acetyl-lysine, and proline as shown in Figure 5A. Lines were obtained by fitting data to eq 2, which illustrates our treatment of the data for Wm and **2a** (initial concentration = $[2a]_i$ or $[Wm]_i$, concentration as a function of time = $[2a]_t$ or $[Wm]_t$), with first order decay constants ($k_{FOD}$) given in Table 2.

$$[\mathbf{2a}]_t = [\mathbf{2a}]_i \exp(-k_{FOD}t) \quad (2)$$

Wm was quite stable in PBS, with a $k_{FOD}$ of 0.012 $h^{-1}$, which corresponded to a half-life of 57.5 h. We repeated with determination of $k_{FOD}$ in the presence of 0.065 mM BSA and determined a $k_{FOB}$ of 0.18 $h^{-1}$, or about 15-fold larger than the $k_{FOD}$ in PBS. The addition of 10 mM proline or 10 mM N-acetyl lysine also accelerated the decay of Wm (see Table 2 for values of $k_{FOD}$). Thus the first order decay constant for Wm was increased by the addition of amine containing molecules or materials, with dramatically higher effects for the secondary amine of proline ($k_{FOD}$ =1.87 $h^{-1}$) compared to the single primary amine of N-acetyl lysine ($k_{FOD}$ =0.30 $h^{-1}$). Addition of 10 mM proline increased $k_{FOD}$ about 100-fold, yielding a half-life of 22 min, which approached the half-lives of Wm in RPM or MEM culture media.[33] RPM and MEM are a mixture of amino acids in the millimolar concentration range. The $k_{FOD}$ was also measured as a function of the pH of the PBS buffer, with values of 0.018 $h^{-1}$ and 0.028 $h^{-1}$ obtained at pH 7.4 and 8.0, respectively. Thus Wm is quite stable in aqueous media (PBS), but its instability was greatly enhanced by biological molecules such as proline, BSA, or N-acetyl lysine.

We also determined the $k_{FOD}$ for the WmC20 derivatives **2a** and **2b** as shown in Figure 5B, with values given in Table 2. The $k_{FOD}$s for **2a** and **2b** were 0.080 $h^{-1}$and 0.0053 $h^{-1}$, respectively, compared to that of Wm which was 0.012 $h^{-1}$. However, the addition of 10 mM proline slightly decreased $k_{FOD}$s for **2a** and **2b**, rather than dramatically increasing the $k_{FOD}$ as was the case for Wm. A similar result was obtained for the addition of 0.065 mM BSA, which had no effect on the $k_{FOD}$ of **2a** and only slightly decreased that of **2b**. Thus Wm modified at C20 by the attachment of 6-N(Me)-hexanoic acid (**2a**) or 6-NH-hexanoic acid (**2b**) was resistant to the increase in the instability produced by the addition of 10 mM proline. It appears that proline enhances the instability of Wm by reacting at the C20 position, since this is the only difference between Wm and **2a** or Wm and **2b**. While the decay of **2a** was not affected by the addition of proline, a peak in addition to Wm was seen on our HPLC chromatograms when **2a** stability was measured. The major peak with **2a** decay was Wm, while the third peek had the same retention time as Wm after reaction with proline (data

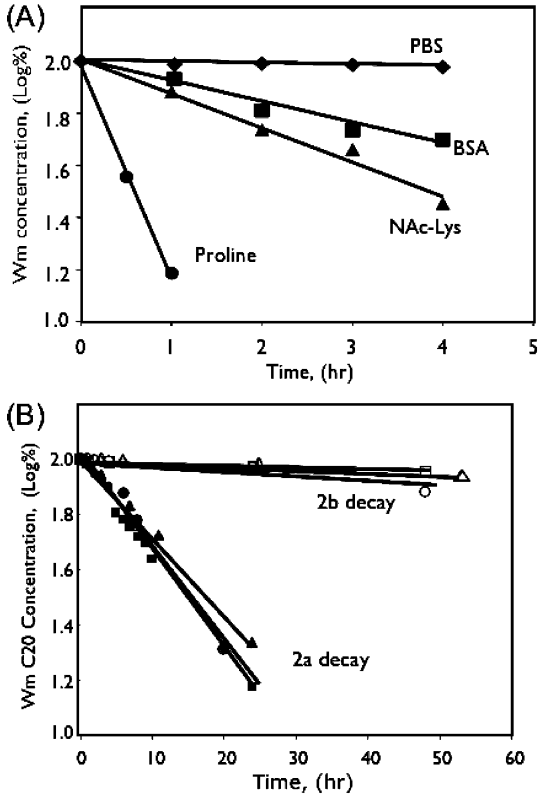


**Figure 5.** Stability of Wm or Wm C20 derivatives. (A) Stability of Wm in PBS with the indicated compounds. (B) Stability of WmC20 derivative **2a** in PBS (●), 10 mM proline (▲), or 0.065 mM BSA (■). Stability of WmC20 derivative **2b** in PBS (○), 10 mM proline (△), or 0.065 mM BSA (□). First order decay constants are given in Table 2.

**Table 2.** First Order Decay (FOD) Constants of Wm and Wm C20 Amines

| compd | conditions | $k_{FOD}$ ($h^{-1}$) (95% confidence intervals) | half-life |
|---|---|---|---|
| Wm | PBS | 0.012 (0.0080-0.016) | 57.8 h |
| Wm | PBS, 0.065 mM BSA | 0.18 (0.16-0.20) | 3.85 h |
| Wm | PBS, 10 mM proline | 1.87 (0.62-3.13) | 22 min |
| Wm | PBS, 10 mM NAcLys | 0.30 (0.23-0.38) | 2.31 h |
| **2a** | PBS | 0.080 (0.071-0.089) | 8.7 h |
| **2a** | PBS, 10 mM Proline | 0.065 (0.0062-0.068) | 10.7 h |
| **2a** | PBS, 0.065 mM BSA | 0.082 (0.075-0.088) | 8.5 h |
| **2b** | PBS | 0.0053 (0.0040-0.0067) | 131 h |
| **2b** | PBS, 10 mM proline | 0.0031 (0.0023-0.0038) | 223 h |
| **2b** | PBS, 0.065 mM BSA | 0.0028 (0.0021-0.0034) | 248 h |
| Wm | RPM[a] | 4.02 | 10.3 min |
| Wm | MEM[a] | 5.04 | 8.3 min |

[a] Values from ref 33.

not shown). This observation suggests that **2a** produced Wm which then reacted with proline.

We then examined the production of Wm from WmC20-N(Me)-hexanoic acid (**2a**) and from WmC20-NH-hexanoic acid (**2b**) in deuterated chloroform by NMR, a neutral organic solvent at ambient temperature as shown in Figure 6. The 0 h spectra of **2a** (Figure 6A) exhibited a small peak at 8.26 ppm, due to the C20 proton of Wm which was present at the initial time point due to trace amounts of self-activation of **2a** to yield Wm during the purification and lyophlization. The peak at 8.26 ppm increased with time, indicating a slow formation of Wm, while a peak at 8.18 ppm, assigned to the C20 proton of **2a**, decreased with time. Thus **2a** decreased 14% and Wm increased 16% over a period of 50 h, which was calculated based on the ratio of integration of these two peaks due to the conversion of **2a** to Wm. The spectra of **2b** (Figure 6B) exhibited peaks at 9.86

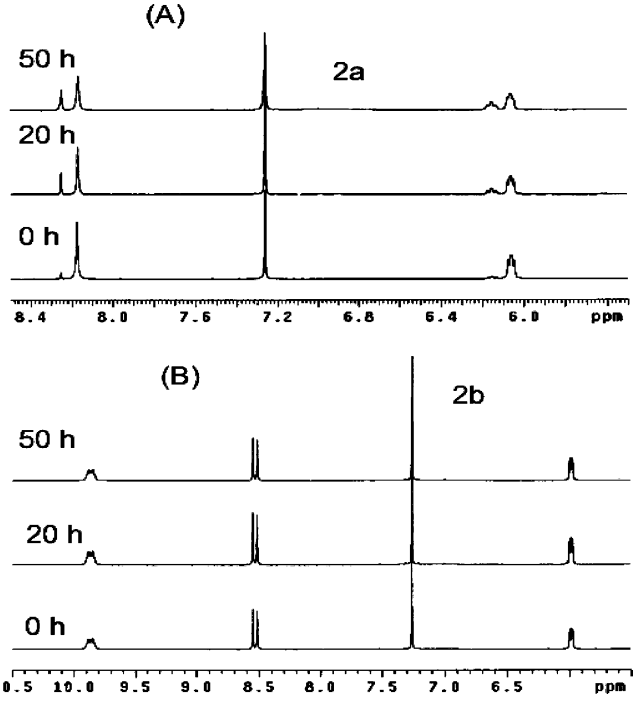


**Figure 6.** Partial NMR spectra for **2a** and **2b** in $CDCl_3$. (A) For **2a**: 8.26 ppm (s, H−C20 of Wm), 8.18 ppm (s, H−C20 of **2a**), 6.16 ppm (m, H−C11 of Wm), 6.07 ppm (m, H−C11 of **2a**). (B) For **2b**: 9.86 ppm (m, C20NH of **2b**), 8.55 ppm (d, $J$ = 13.9 Hz, H−C20 of **2b**), 5.99 ppm (m, H−C11 of **2b**).

ppm (m, C20NH), 8.55 ppm (d, $J$ = 13.9 Hz, H−C20), and 5.99 ppm (m, H−C11), respectively, and were independent of time. The ability of **2a** but not **2b** to generate Wm occurs both in PBS at pH 6.8 and in chloroform.

Finally, we sought to confirm our HPLC and NMR results that WmC20 derivatives employing 6-N-methylaminohexanoic acid linker (**2a**, **4a**, **5a**) would generate Wm, while WmC20 derivatives employing the 6-aminohexanoic acid linker (**2b**, **4b**, **5b**) could not do so, using a PI3K enzyme assay. We therefore incubated all six compounds for 3 h at 37 °C in PBS to allow significant Wm generation, the complete time course for which is shown in Figure 4. PBS plus Wm C20 derivative was then added to PI3K to assess its ability to inhibit PI3K activity. $IC_{50}$s for each compound are given in Table 3. Also shown are the percentages of Wm released from the WmC20 adduct at the 3 h time point from Figure 4. WmC20 derivatives employing the 6-N-methylaminohexanoic acid linker (**2a**, **4a**, **5a**) inhibited PI3K with $IC_{50}$s that were 3- to 32-fold higher than Wm, while the corresponding WmC20 derivatives employing the 6-N-aminohexanoic acid linker (**2b**, **4b**, **5b**) did not inhibit PI3K at 1000 nM or at $IC_{50}$ at least 250-fold higher than Wm. Both HPLC assays and a PI3K enzyme inhibition assay indicate that Wm was produced from three WmC20 derivatives employing the 6-N-methylaminohexanoic acid linker but not from those employing the 6-aminohexanoic acid linker. The relative potency of WmC20 derivatives employing the 6-(N-methylamino)-hexanoic acid linker was similar as assayed by HPLC and as inhibitors of Wm (**4a** > **2a** > **5a**).

## Discussion

Wm derivatives were synthesized by reacting the C20 of Wm with 6-N-methylaminohexanoic acid or 6-aminohexanoic acid (**2a**, **2b**), followed by the conjugation of **2a** and **2b** to amine groups of proteins or beads. Wm compounds made with 6-(N-methylamino)-hexanoic acid (**2a**, **4a**, **5a**, **6a**) generated Wm

**Table 3.** Inhibition of PI3K by Wm C20 Derivatives and Conjugate

| | bioassay: PI3K $IC_{50}$ (nM) (95% confidence intervals) | $IC_{50}$Wm/$IC_{50}$ Wm-derivative | Wm generation (%) by HPLC (3 h, 37 °C) |
|---|---|---|---|
| Wm (**1**) | 4.3 ± 1.3 (2.5-7.4) | NA | NA |
| WmC20-N(Me)-hexanoic acid (**2a**) | 36.7 ±1.0 (34.5-39.0) | 0.117 | 10.4 |
| WmC20-NH-hexanoic acid (**2b**) | >1000 | <0.004 | ND |
| WmC20-N(Me)-hexanoate-BSA (**4a**) | 14.8 ± 1.1 (12.6-17.4) | 0.29 | 16.5 |
| WmC20-NH-hexanoate-BSA (**4b** ) | >1000 | <0.004 | ND |
| WmC20-N(Me)-hexanoate-IgG (**5a**) | 129.9 (104.4 to 161.5) | 0.033 | 3.5 |
| WmC20-NH-hexanoate-IgG (**5b**) | >1000 | <0.004 | ND |

(Figures 3 and 4) while the corresponding compounds made with 6-aminohexanoic acid (**2b**, **4b**, **5b**, **6b**) did not. The production of Wm correlated with the inhibition of PI3K enzyme activity; WmC20-N(Me)-hexanoic based compounds inhibited PI3K while WmC20-NH-hexanoic based compounds failed to do so (Table 3). The pattern of inhibition of PI3K we obtained was consistent with the observations of Norman.[13,12] Norman obtained an $IC_{50}$ of 80 nM for a Wm reacted at C20 with the secondary amine, diethylamine, compared to an $IC_{50}$ of 36.7 nM we obtained for Wm reacted with the secondary amine, 6-(*N*-methylamino)-hexanoic acid, see Table 3 (**2a**). Norman obtained an $IC_{50}$ of greater than 500 nM for Wm reacted at C20 with the primary amine, ethylamine, which compared well with the $IC_{50}$ of greater than 1000 nM we obtained for Wm reacted with the primary amine 6-aminohexanoic acid (**2b**). In both studies the $IC_{50}$ for Wm was 4.3 nM.

We propose that the WmC20-N(Me)-hexanoic acid derivative (**2a**), which cannot form the hydrogen bonded ring structure (Figure 2), inhibited PI3K by generating Wm through an intramolecular attack of the C6 hydroxyl group (Figure 1, solid arrow). The WmC20-NH-hexanoic acid derivative (**2b**) failed to inhibit PI3K because it was stabilized by hydrogen bonded ring, so that Wm formation was either nonexistent or too slow to be detected. The control of the intramolecular attack by the hydrogen bonded ring determined whether Wm was generated, not only with the low molecular weight WmC20 N(Me)-hexanoic acid (**2a**) or WmC20 NH-hexanoic (**2b**) derivatives but also when these derivatives were covalently coupled to amine groups, to yield the high molecular weight Wm conjugates (**4a**, **b**−**6a**, **b**). The intramolecular attack was due to a combination of features of the WmC20 derivatives and conjugates including the enamine linkage of the nitrogen at C20, the lack of the stabilizing hydrogen bond with carbonyl at C3, the proximity of enol hydroxyl group at C6, and perhaps other features of the Wm molecule. The intramolecular mechanism for Wm generation was supported by the production of Wm from **2a** in chloroform (Figure 6), a nonaqueous solvent without added acid or base. We know of no other case where a pharmacologically active substance can be covalently conjugated to a protein and regenerate itself under physiological conditions through an enzyme independent, solvent independent, intramolecular attack.

The slow increase and decrease in Wm concentration when WmC20 6-N(Me)-hexanoic based compounds were incubated in PBS (Figure 4) requires considerable stability of Wm and seemed to be inconsistent with the half-life of 8−13 min reported for Wm in tissue culture media.[33] We therefore measured the stability of Wm as first order decay constants in PBS and in PBS with amine bearing molecules added (Table 2). Wm was highly stable in PBS, but its stability was decreased with the addition of BSA, N-acetyl-lysine, or proline. The $k_{FOD}$ obtained with 10 mM proline of 1.87 $h^{-1}$ was about half the reported values for a similar constant for Wm stability in tissue culture media.[33] In our hands the half-life of Wm ranged from 57.5 h (in PBS) to 22 min (in PBS plus 10 mM proline). Wm can be highly stable or highly unstable in an aqueous environment depending on the presence of biological molecules such as amino acids or proteins.

Proposing that WmC20-N(Me)-hexanoic acid based compounds are self-activating requires that they be inactive as inhibitors of PI3K and converted to the active inhibitor (Wm). However, because WmC20-N(Me)-hexanoic acid and compounds made using it generate Wm under conditions of the PI3K assay (phosphate buffer), the ability to demonstrate that they are not inhibitors of PI3K is somewhat limited. The evidence that WmC20-N(Me)-hexanoic acid based compounds fail to inhibit PI3K until they generate Wm has been inferred from different types of experiments: (i) WmC20-N(Me)-hexanoic acid based compounds, but not WmC20-NH-hexanoic acid based compounds, generated Wm with our HPLC assay (Figure 3), (ii) WmC20-N(Me)-hexanoic acid, but not WmC20-NH-hexanoic acid, generated Wm in chloroform evident by NMR, (iii) WmC20-N(Me)-hexanoic acid based compounds, but not WmC20-NH-hexanoic based compounds, inhibited PI3K. The inhibition of PI3K through Wm generation is also supported by the X-ray crystal structure of PI3K,[2] which appears not to allow WmC20 based compounds with very large modifications at C20 (**4a**, **5a**) to fit into the ATP binding pocket as Wm does.

Our studies have several implications for the use of Wm in signal transduction research and for the development of low molecular weight Wm derivatives and high molecular weight Wm conjugates as pharmaceuticals. First, our observation that Wm is stable in PBS but not in the presence of proline, together with observations about its instability in tissue culture media,[33] may result in new experimental designs or a reinterpretation of older literature where Wm was used to evaluate the function of the PI3 kinase pathway through its inhibition. Second, the pharmacological potency of Wm based compounds may depend on their ability to generate Wm and survive in biological media, in addition to the usual parameters determining drug potency, such as the ability of the synthesized compound to bind a molecular target (PI3K) or the ability to undergo membrane translocation. Wm is a highly unusual compound that is rapidly degraded in biological media, whose derivatives vary widely in their stability in aqueous media, and whose degradation is influenced profoundly by common substances in biological systems. Understanding these features of Wm, Wm derivatives, or Wm conjugates may lead to new dosing schedules that recognize these specific features. Third, the chemistry we have described permits the attachment of the N-hydroxysuccinimide ester of WmC20-N(Me)-hexanoic acid (**3a**) to the amines of any amine bearing targeting biomolecule (antibody, peptide, polysaccharide) or solid phase. This provides a general method of designing self-activating WmC20 conjugates that maybe useful as tissue or cell selective PI3K inhibitors. The development of targeted PI3K inhibitors may be essential for the development of successful PI3K inhibiting pharmaceuticals

because of the enzyme's broad distribution and numerous essential roles.

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**Supporting Information Available:** Information on purity of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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