17β-HYDROXY-16α-[^{125}I]IODOWORTMANNIN, A SENSITIVE LABELING AGENT FOR PI 3-KINASES

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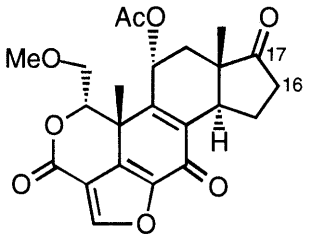
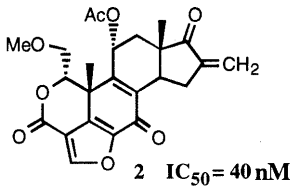
To detect phosphatidylinositol (PI) 3-kinases with high sensitivity, we designed and prepared a radiolabeled derivative of wortmannin (**1**), a potent inhibitor of PI 3-kinases. The synthesized derivative, 17β-hydroxy-16α-[^{125}I]iodowortmannin (**7**), showed an IC_{50} that was 100-fold higher than that of wortmannin itself and which bound to catalytic subunits of PI 3-kinases.

KEY WORDS wortmannin; PI 3-kinase; radiolabeling; signal transduction

Phosphatidylinositol (PI) 3-kinases are important components of intracellular signal transduction systems that have been implicated in cell growth and oncogenesis.[1] PI 3-kinases phosphorylate at the D-3 positions of the inositol ring in phosphatidylinositol, phosphatidylinositol-4-phosphate (PI-4-P), and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) *in vitro* and can be activated by various types of agonist-stimulated receptors, including growth factor receptor tyrosine kinases (*e.g.* platelet-derived growth factor (PDGF) receptor and insulin receptor) and GTP-binding protein (G protein)-coupled receptors. The roles of 3-phosphorylated phosphoinositides are presently unclear, but they might be putative intracellular second messengers. This hypothesis has been suggested by the fact that phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) was found to activate some isoforms of protein kinase C *in vitro*.[2]



wortmannin (1) IC_{50} = 3 nM



2 IC_{50} = 40 nM

Several isoforms of this enzyme have been reported, one of which, referred to as the "conventional isoform" in this paper, is a heterodimer consisting of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110), and can be activated by binding directly to agonist-stimulated receptor tyrosine kinases through specifically autophosphorylated tyrosine residues.[1] Recently, another isoform has been discovered[3] that is regulated by the βγ subunits of heterotrimeric G protein, which would be released upon stimulation of G protein-coupled receptors.

Wortmannin (**1**) is a potent and specific inhibitor of PI 3-kinases (IC_{50} = 3 nM)[4] that is believed to bind to the enzymes in an irreversible, presumably covalent manner.[4b] Its structure is similar to that of steroids and includes a strained and highly reactive furanocyclohexadienone lactone moiety, which seems to be the putative binding site to the kinase, presumably through its cysteine or lysine residue. Wortmannin (**1**) has been used to elucidate the role(s) of PI 3-kinase in cells and signal transduction pathways,[1b] but the possibility cannot be excluded that wortmannin might bind to other unknown component(s) in cells and that such component(s) might play significant roles in signal transduction. If sensitive radiolabeled derivatives of **1** are prepared, PI 3-kinases could be easily detected on SDS-PAGE, and novel wortmannin-binding component(s) might be discovered, which would lead to a clearer understanding of the roles of PI 3-kinases in cells and of the details of signal transduction pathways. Therefore, we intended to design and prepare a radiolabeled derivative of wortmannin, such as 17β-hydroxy-16α-[^{125}I]iodowortmannin (**7**). We report here the synthesis and evaluation of (**7**) for binding to PI 3-kinases.

Iodine-125 was selected for radioisotope labeling because of its high relative radioactivity and ease of detection. In addition, Yano *et al.* reported studies on the structure-activity relationships of (**1**),[4b] in which the 16-

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exo-methylene derivative (**2**) showed a slight loss of inhibitory activity (IC_{50}= 40 nM). Another group showed that the 17-hydroxy derivative retained inhibitory activity (IC_{50}= 5 nM).[5] These results suggested that the five-membered ring (D-ring in steroid nomenclature) should be structurally modified for radioisotope labeling. Based on these findings, as well as on the relatively easy manipulation of the D-ring, we designed 17β-hydroxy-16α-[^{125}I]iodowortmannin (**7**) as a sensitive labeling agent for PI 3-kinases.

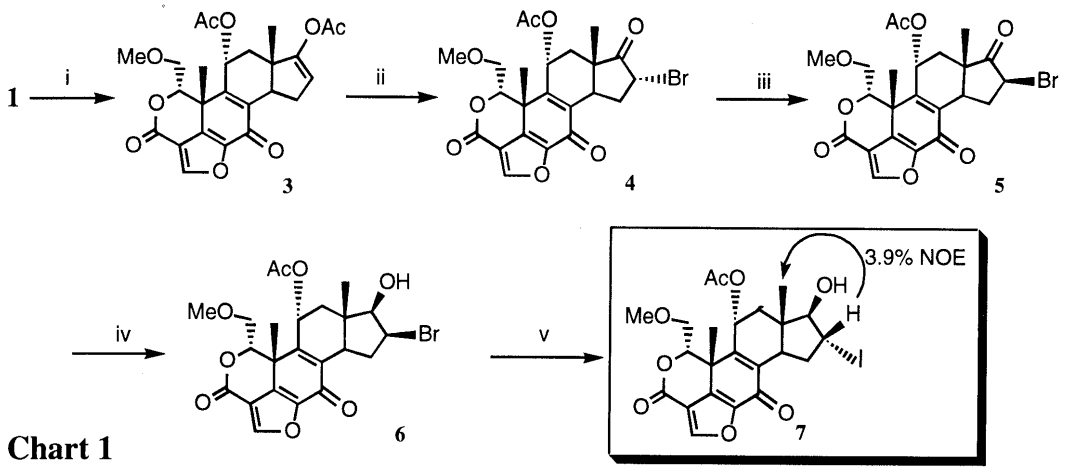

Chart 1

Reagents and conditions: 1) CH_2=CCH_3(OAc), cat. conc. H_2SO_4 (15% and **1** recovery 82%); ii) NBS, 2N H_2SO_4 aq, CH_2Cl_2-^{t}BuOH (83%); iii) excess LiBr, 2-butanone, room temperature, overnight (51% and **4** recovery 48%); iv) BH_3·THF, 0 °C, 3h (86%); v) NaI, PhCOOH, $Na_2S_2O_3$, 2-butanone, reflux (73%).

The synthesis was carried out according to the procedure reported in the estradiol system.[6] 16α-Bromowortmannin (**4**) was synthesized using the method reported by Haefliger *et al.*[7] 16β-Bromo ketone (**5**)[8] was obtained by epimerization of **4** with excess amounts of LiBr under equilibration (α:β = 49:51), and then its carbonyl group at the 17-position was reduced upon treatment with 3 mol eq of BH_3·THF in THF at 0 °C to give 16β-bromo-17β-hydroxywortmannin (**6**)[8] from **5** at a yield of 86%. Halogen exchange on **6** was performed with 1.2 eq of NaI in 2-butanone at reflux in the presence of benzoic acid and $Na_2S_2O_3$, giving iodohydrin (**7**)[8] in 73% yield with full inversion at the 16-position. The resulting **7** was assayed for its inhibitory activity against PI 3-kinase *in vitro* and showed an IC_{50} (IC_{50}=300 nM) that was 100-fold higher than that of wortmannin itself. The significant loss of inhibitory activity might result from a bulky group such as the iodine atom at the 16-position, which would lead to a reduction of the interaction with PI 3-kinase. 17β-Hydroxy-16α-[^{125}I]iodowortmannin, however, was found to be a useful radiolabeled probe for PI 3-kinases, as discussed below.

Radiolabeled **7** was synthesized as follows: 10μl of 1% $Na_2S_2O_3$ solution in 90% aqueous MeOH was added to 300μl of Reactivial^{TM} (Pierce Co.) , followed by 5μl of 0.1% benzoic acid solution in MeOH and 10μl of Na[^{125}I] solution in 0.1N NaOH aq. (1mCi/10μl, NEN-DuPont). The solvent was removed and to the resulting residue was added a solution of bromohydrin (**6**) in CH_3CN (10μg/10μl). After 2 h of heating at 120 °C in a sealed tube, the reaction mixture was diluted with 10μl CH_3CN, loaded directly on HPLC (J'sphere H80, YMC Co., Ltd.) and eluted with CH_3CN-H_2O (50/50, w/w). Radioactive fractions were collected with a retention time of about 14.0 min., which was identical to that of **7** prepared in a preliminary study. The radioactive yield of **7** was *ca.* 60%. Collected fractions were lyophilized and stored at -25 °C.

We next examined the binding affinity of radioactive **7** to the conventional isoform of PI 3-kinases. Binding studies were performed as follows: **7** was incubated with partially purified conventional PI 3-kinase from rat liver. After the addition of sample buffer, the mixture was subjected to SDS-PAGE. The band corresponding to p110 was detected on SDS-PAGE, but disappeared when preincubated with non-labeled wortmannin. p85 Subunit was not detected by this agent. These results are in agreement with the hypothesis that wortmannin (**1**) binds to the p110

subunit, presumably covalently. Moreover, using this labeled derivative, we succeeded in identifying a novel isoform of PI 3-kinases[9] from rat liver, which was electrophoresed at about 100kDa.

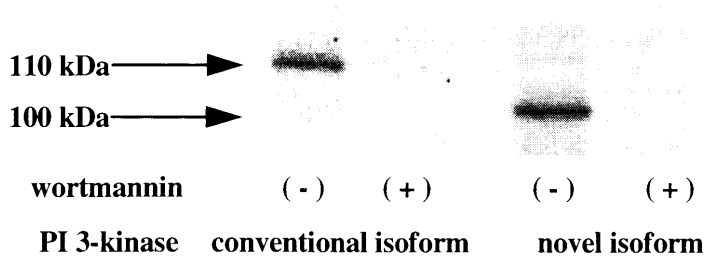

Fig. 1

The conventional and novel isoforms[9] of PI 3-kinases from rat liver were incubated with or without 0.1 μM wortmannin for 10 min. before the addition of **7**. The radiolabeled peptides thus obtained were separated by SDS-PAGE. The autoradiogram used to localize the labeled peptides is shown.

In summary, we designed and prepared a radiolabeled derivative of the PI 3-kinase inhibitor wortmannin (**1**) for use as a probe for these enzymes. The binding ability of **7** to these enzymes was decreased, but the catalytic subunit of these enzymes was adequately detected on SDS-PAGE by using 17β-hydroxy-16α-[^{125}I]iodowortmannin. Using this tool, we are now developing a method to identify novel isoforms of PI 3-kinases which play a role in the cell signal transduction pathway. In addition, this labeled compound could be used to search for the binding site on PI 3-kinases, as in the photoaffinity-labeling technique.

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