Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 245-249

FARNESYL-DERIVED INHIBITORS OF *RAS* FARNESYL TRANSFERASE

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Received October 3, 1995

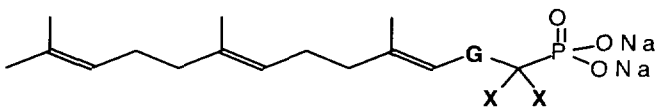
Summary: The synthesis and evaluation of farnesyl-derived inhibitors of *ras* farnesyl transferase are presented. Evaluation of inhibitors of farnesyl transferase and comparison with the previously described inhibitor was accomplished using purified enzyme and Amersham's Farnesyl:Transferase enzyme assay kit. These results show an order of magnitude increase in inhibitory activity for β-ketophosphonic acid over β-hydroxyphosphonic acid . Incorporation of fluorines in α-position, led to an increase in inhibitory activity over the nonfluorinated analogues.

Mammalian *ras* genes encode for GTP-binding proteins involved in signal transduction pathways for cell growth and differentiation(1-3). The occurrence of *ras* oncogenes in 30% of human tumors (4) along with the elucidation of the posttranslational steps required for activity of the *ras* proteins (5) has made inhibition of *ras* protein function an emerging target for drug design for tumors in which *ras* may contribute to tumor formation (6).

Membrane localization is required for activity of the oncogenic *ras* proteins. Localization is dependent on three posttranslational modifications; farnesylation, proteolysis, and methyl esterification. Farnesylation of a specific cysteine residue near the carboxy terminus, such as Cys186 of Ha-p21ras, is catalyzed by farnesyl transferase using farnesyl pyrophosphate as the isoprenoid donor. A mechanistic study of farnesyl transferase has shown a random sequential order for the binding of substrates for this Mg^{2+} and Zn^{2+} dependent enzyme (7). Proteolytic removal of three carboxyl-terminal residues and methyl esterification of the new carboxyl-

0006-291X/95 $12.00

terminus farnesylated cysteine provides a protein capable of membrane localization. Interference with membrane localization by inhibition of any of these three steps may provide a means for intervention with a chemotherapeutic agent (6). The sequence requirement for peptidyl substrates of farnesyl transferase has been delineated (8) and a number of small peptide-based and peptidomimetic inhibitors of that enzyme have been prepared (9-11). Several of these peptide-derived inhibitors decrease the amount of *ras* protein processed leading to an increase in unprocessed cytosolic *ras* protein which has a negative inhibitory effect on membrane bound mutant *ras* protein (12). Proteins other than *ras* are isoprenyated *in vivo*; most are modified by a C-20 geranylgeranyl moiety by a transferase similar to farnesyl transferase. Both of these transferases have a common α-subunit, however, the β-subunit of each is distinct and determines whether the transferase will catalyze the transfer of a farnesyl or a geranylgeranyl group. A specific inhibitor of farnesyl transferase which minimized interference with other isoprenylations would be most useful. To avoid the metabolic and bioavailability problems often associated with peptide-based inhibitors, as well as interference with the function of geranylgeranyl modified proteins, we synthesized ketones **1** and **2** and alcohols **7** and **8**, as mimetics of farnesyl pyrohposphate and tested their potential as inhibitors of farnesyl transferase.

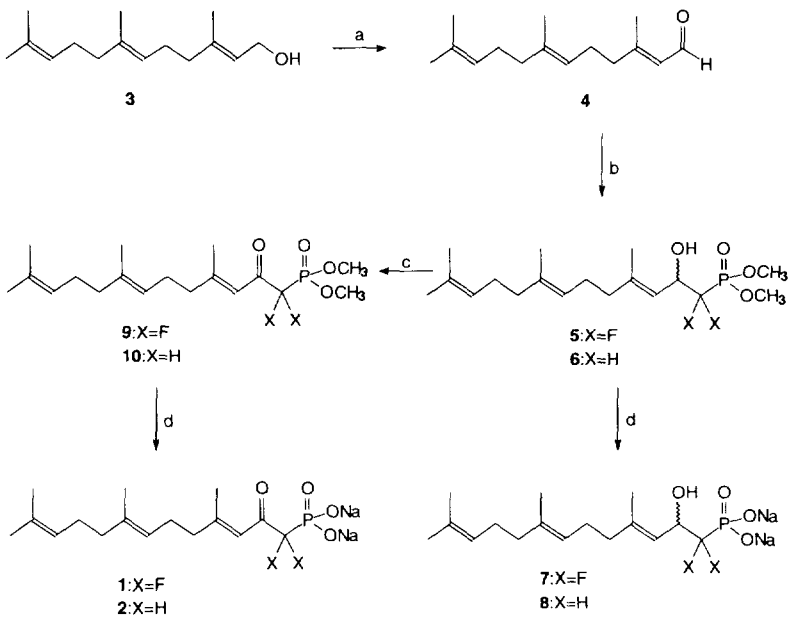


1: X = F, G = CO
2: X = H, G = CO

7: X = F, G = CHOH
8: X = H, G = CHOH

MATERIALS AND METHODS

These inhibitors were prepared as shown in Scheme I (13). Farnesal **4** was prepared, reproducibly and in essentially pure form by oxidation of farnesol **3** with $BaMnO_4$ (14). The addition of lithio dimethyl methylphosphonate (15) to **3** proceeded smoothly to provide dimethyl β-hydroxyphosphonate **6**. The reaction of lithio dimethyl difluoromethylphosphonate (16) with **3**, however, required careful monitoring of the reaction temperature both during the formation of the anion and during the addition of the aldehyde. Reaction temperatures above -72°C caused an exothermic decomposition of the reagent that led to recovered starting aldehyde and greatly decreased yields of dimethyl β-hydroxyphosphonate **5**. Oxidation of **5** by the method of Swern using trifluoroacetic anhydride (17) gave dimethyl β-ketophosphonate **9**. Oxidation of dimethyl β-hydroxyphosphonate **6** with $BaMnO_4$ (14) provided dimethyl β-ketophosphonate **10**. The dimethyl β-ketophosphonates **9** and **10** and the dimethyl β-hydroxyphosphonates **5** and **6** were demethylated and purified according to the method of Biller (18) to give β-ketophosphonic acids **1** and **2** and β-hydroxyphosphonic acids **7** and **8**, respectively. Evaluation of compounds and comparison with previously described inhibitor **11** (7) was performed as described below.



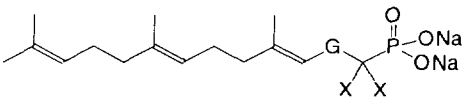
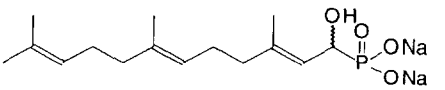
***Scheme I.* Synthesis of Inhibitors**

Reagents and Conditions: **a**) $BaMnO_4$, CH_2Cl_2; 5 days; **b**) when X is H, dimethyl methylphosphonate, LDA, THF, -70°C, and when X is F, dimethyl difluoromethylphosphonate, LDA, THF, <-72°C; **c**) when X is H, $BaMnO_4$, CH_2Cl_2, 5 days, and when X is F, 1) DMSO, TFAA, CH_2Cl_2, 2) NEt_3; **d**) TMSI, collidine, CH_2Cl_2, 0°C.

Enzyme Purification: 100 g of pig kidney (Pel-freez Biologicals, Ark.) was homogenized for 2 minutes in buffer A (100 mM Tris pH 7.5, containing 1 mM of each EDTA, EGTA, DTT and 1μg/mL each of the protease inhibitors leupeptin, antipain, pepstatin, and 0.1 mM caproic acid and 0.5 mM PMSF). The homogenate was spun at 10,000 x g for 60 minutes to get the soluble fraction. The supernatant was made 50% with ammonium sulfate and stirred at 4°C for 30 minutes and spun at 12,000 x g for 30 minutes to get the pellet. The pellet was dissolved in buffer B (50 mM Tris pH 7.5 containing 1 mM DTT and 20 μM $ZnCl_2$) and dialyzed to recover the enzyme. The ammonium sulfate precipitated enzyme was further purified on a column Q (Q Sepharose, Pharmacia) and eluted with 200-800 mM NaCl gradient in buffer B. Partially purified enzyme eluted with around 500 mM NaCl was used as source of enzyme. The enzyme stored at -70°C after dialysis against buffer B is stable under these conditions for several months.

Assays: The assay is based on the scintillation proximity principle. A human lamin-B carboxyl-terminus sequence peptide linked to biotin is ^{3}H-farnesylated at the cysteine residue by farnesyl:transferase. The resultant complex is captured by a streptavidin-linked SPA bead. In a 100 μl total reaction volume, the test compound was added in 10 μL of solvent, usually DMSO, then 70 μL of the reaction mixture containing 10 μL of 1% Triton X-100, 20 μL of 1:100 diluted ^{3}H-farnesyldiphosphate (0. 2 μCi), 20 μL of assay buffer (50 mM HEPES, 30 mM MgCl2, 20 mM KCl, 5 mM DTT, and 0.01% Triton X-100), and 20 μL of 0.5 μM Biotin-Lamin B peptide in buffer pH 7.5 (50 mM HEPES, 25 mM Na_2HPO_4, 20 mM KCl, 5 mM DTT, and 0.01% Triton X-100) was added. This mixture was allowed to stand at 37°C for 5 minutes. The reaction was started by adding of 20 μL of enzyme (~3 μg of protein). The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 150 μL of the stop/bead reagent. The samples were counted in a Packard Tri-Carb 1900 CA scintillation counter.

Table I. **Inhibition of Farnesyl Transferase.**

			Inhibition of Farnesyl Transferase
compound	G	X	IC_{50} (μM)
1	-C(O)-	F	0.35
2	-C(O)-	H	1.56
7	-CH(OH)-	F	0.78
8	-CH(OH)-	H	11.5
 **11**			0.15

RESULTS AND DISCUSSION

The inhibitory values given in Table I suggest that all of the farnesyl phosphonic acid derivatives prepared were good inhibitors of farnesyl:transferase. These results show almost an order of magnitude increase in inhibitory activity for β-ketophosphonic acid **2** over β-hydroxyphosphonic acid **8**. Incorporation of fluorines at the α-position, i.e. β-ketophosphonic acid **1** and β-hydroxyphosphonic acid **7,** led to a 5-15 fold increase in inhibition over the non-fluorinated analogues. It can be seen that ketone **1**, the most electrophilic ketone, is the most potent inhibitor showing comparable inhibition to the literature standard **11**. The next most potent inhibitor is α,α-difloro alcohol **7**. These facts are most consistent with the pKa of the phosponate moiety controlling the inhibitory activities of the compounds prepared. α,α-Difloro substitution is well known to increase the acidity of phosphonate group and , thus more closely mimic a phosphate moiety. These results further suggest that farnesyl derived inhibitors may have potential as chemotherapeutic agents.

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