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SYNTHESIS AND PREPARATION OF AN AFFINITY CHROMATOGRAPHY COLUMN FOR THE PURIFICATION OF CYTOSOLIC PHOSPHOLIPASE A_2

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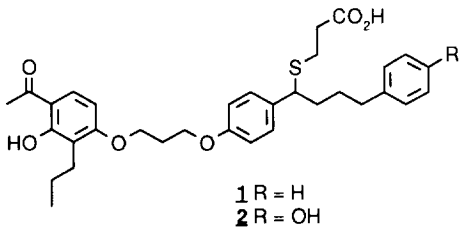
Abstract: An affinity column has been developed to provide a single step purification of the human recombinant baculovirus overexpressed $cPLA_2$. The affinity matrix is based on the chemical structure of a potent inhibitor of $cPLA_2$.

Eicosanoids are highly active substances with diverse biological actions. Elevated levels of eicosanoids in response to a variety of stimuli have been implicated in the pathology of many inflammatory diseases.[1] The rate-limiting step in the generation of eicosanoids is the mobilization of arachidonic acid (AA) in many cell types.[2] Among the several proposed enzymatic pathways, cytosolic phospholipase A_2 ($cPLA_2$) is thought to be the enzyme responsible for the release of free AA.[3]

$cPLA_2$, an 85 kD protein is hormonally regulated in cells[4] and is activated at physiological concentrations (sub-micromolar) of Ca^{2+} in the stimulated cells. This enzyme preferentially catalyzes the hydrolysis of AA ester at the *sn*-2 position of the phospholipid, which is further metabolized by lipoxygenase and cyclooxygenase to form proinflammatory lipid mediators.[5] The development of selective inhibitors of $cPLA_2$ has been an intense area of research, not only to dissect the AA metabolic pathways but mainly to use such compounds as potential therapeutic agents in the treatment of inflammation.

Recently we disclosed a series of potent $cPLA_2$ inhibitors exemplified by **1**.[6] Compound **1** has an IC_{50} of approximately 1 μM in cell free assay and in U937 cells where it inhibits the $cPLA_2$ dependent AA release. It is selective for the inhibition of $cPLA_2$ versus the low molecular weight secreted forms, which include the pancreatic and synovial enzymes.[7] In this paper we report the synthesis of an affinity column based on this class of inhibitors which allowed the purification of human recombinant baculovirus overexpressed $cPLA_2$ in a single step.[8]

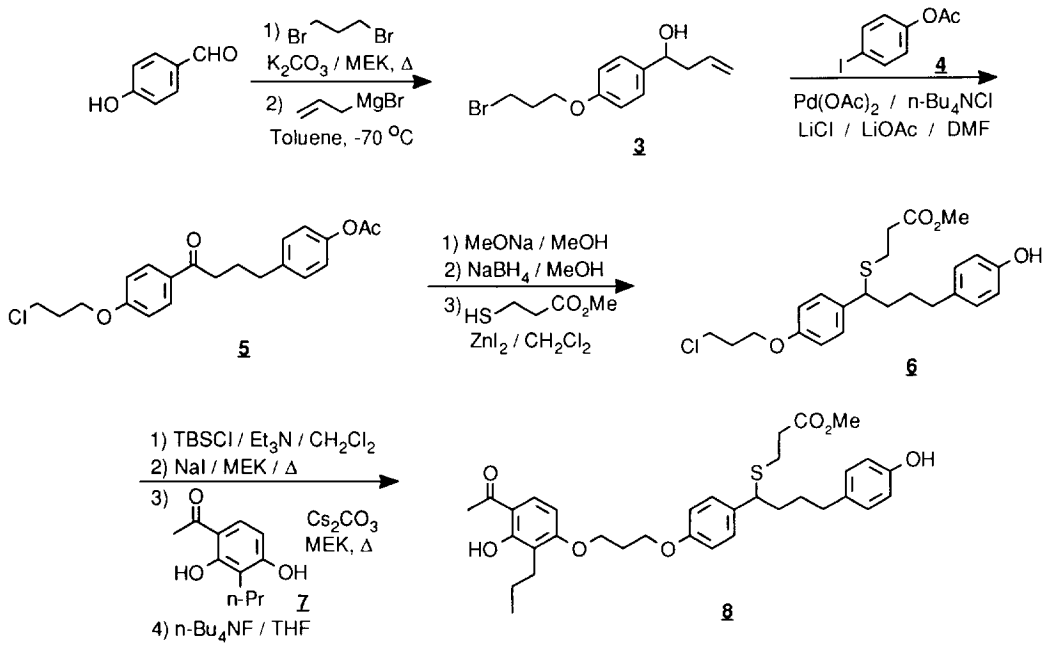


1 R = H
2 R = OH

For the preparation of the affinity column, various attachment points on compound **1** were considered. Based on the structure-activity relationship of this class of compounds the preferred point of coupling to the matrix was at the terminal phenyl ring. For example, various substituents on this phenyl ring are well tolerated including a hydroxyl group which can serve as an attachment point.

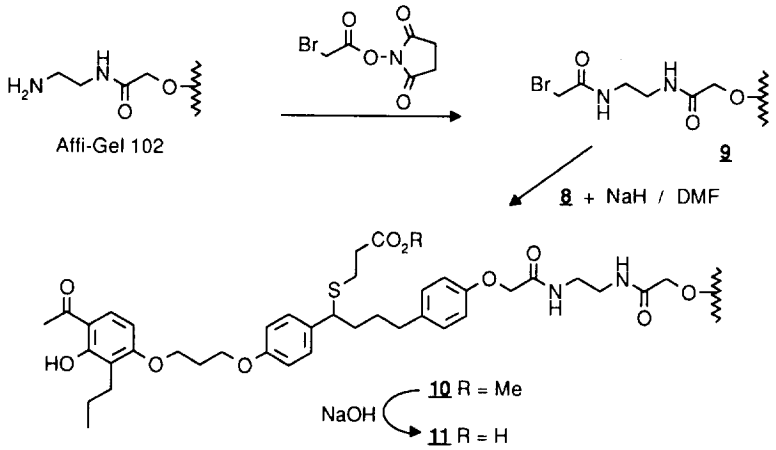
The synthesis of the ligand was achieved in ten steps from 4-hydroxybenzaldehyde (Scheme 1). Alkylation with 1,3-dibromopropane followed by the addition of allyl magnesium bromide to the aldehyde gave the homoallylic alcohol **3**. The Heck reaction[9] was used to couple the homoallylic alcohol **3** with 4-acetoxyphenyliodide **4** to give the required acylated precursor **5**. During this step the bromopropyl ether was converted to a chloropropyl ether due to the presence of the chloride salts required for this catalytic transformation. After deprotection of the phenol and reduction of the ketone of compound **5** with sodium borohydride, the 3-mercaptopropionate chain was introduced by activation of the benzylic alcohol with zinc iodide[10] to give the compound **6**. Protection of the phenol with TBSCl followed by treatment with sodium iodide gave the iodopropyl ether required for the regioselective alkylation of the 3-*n*-propylresacetophenone **7**.[11] Upon treatment of the resulting ether with fluoride ions, the 4-hydroxyphenyl analog **8** was obtained.

Scheme 1



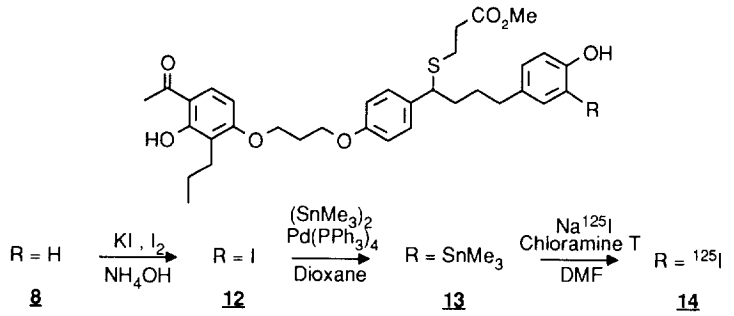
In order to modify the functionality of the polymeric support Affi-Gel 102[12] a spacer unit was then introduced. Affi-Gel 102 was derivatized to accept a ligand with a nucleophilic coupling site such as a phenol. This derivatization consisted of converting the primary amine of the matrix into a bromoacetamide[13] as shown in scheme 2. The ligand was then coupled to the matrix by the displacement of the bromide with the anion derived from compound **8**.[14] The resulting gel was treated with sodium hydroxide for 20 hours and washed thoroughly with phosphate-buffered saline.

Scheme 2



To determine the coupling efficiency, varying amounts of ligand **8** mixed with a radio-iodinated analog **14** were allowed to react with the bromoacetamide derivative of Affi-Gel 102 in a similar manner. The required radio-iodinated analog **14** was prepared in three steps from the phenol **8** (Scheme 3), *ortho* iodination[15] followed by a palladium catalyzed stannylation[16] and treatment of the resulting stannyl derivative with radioactive sodium iodide and chloramine T.[17] A portion of the resulting gel was then counted in a gamma counter. The most efficient coupling ratio was found to be 1 mg of inhibitor **8** per 1 mL of derivatized Affi-Gel 102. The affinity matrix obtained by this method was found to be stable at 4 °C for at least one year.

Scheme 3



We have recently achieved high level expression of active human $cPLA_2$ in insect cells (Sf9) using a recombinant baculovirus.[18] When filtered cytosolic proteins from an Sf9 cell preparation were applied to the affinity column, 95 % of the total $cPLA_2$ activity was retained on the column whereas bromoacetamide-derivatized Affi-Gel 102 did not retain any activity. Similar results were obtained with the cytosol from human monocyte U937 cells, from which purification and characterization have been reported previously.[19] Elution of the active $cPLA_2$ from the column was achieved with a gradient of 0-2 % CHAPS in 50 mM Tris-HCl, pH 7.4.[8]

For preparative purification, up to 2-3 mg of purified $cPLA_2$ can be obtained in a single step from the cytosol using 100 mL of affinity matrix.

In summary, we have prepared an efficient $cPLA_2$ affinity chromatography column by modifying the substitution of a $cPLA_2$ inhibitor and coupling of this analog to a matrix. The high capacity and efficiency of the affinity matrix thus obtained provide rapid purification of $cPLA_2$ in milligram quantities which can be used for structural and biochemical studies.

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