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ENZYMATIC SYNTHESIS OF A MODIFIED PHOSPHOLIPID AND ITS EVALUATION AS A SUBSTRATE FOR *B. CEREUS* PHOSPHOLIPASE C

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Abstract. The novel phospholipid **2**, which bears a *tert*-butyl moiety in place of the natural trimethyl ammonium group of phosphatidylcholine, has been enzymatically synthesized via a transphosphatidylation reaction mediated by phospholipase D. The change from the choline headgroup in **1** to the *tert*-butyl group in **2** reduced the efficiency of hydrolysis by the phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* by a factor of greater than 10^3.

The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc}) is a 28.5 kDa monomeric enzyme that possesses three zinc ions in its active site.[1] This bacterial enzyme has been used as a probe of the signal transduction pathway in mammalian systems and has been found to have immunological similarities with its mammalian counterpart.[2,3] The mechanism by which PLC_{Bc} catalyzes the hydrolysis of phospholipids to provide diacylglycerol and a phosphorylated headgroup has been the subject of a number of investigations.[4-6] This interest in PLC_{Bc} stems from the novel reaction mechanism the enzyme appears to invoke, in which all three zinc ions are used in binding and catalysis.

One focus of our investigations has been in discerning what features of a phospholipid such as **1** are necessary for binding and turnover by PLC_{Bc}. Structure-activity relationship studies on the phosphate moiety have shown that replacement of the bridging oxygen in the scissile phosphate bond with carbon or nitrogen leads to nonhydrolyzable inhibitors, having modest K_is on the order of 100 μM.[7] Substitution on the side chains of the glycerol moiety of phospholipids may have an equally dramatic effect on activity. For example, replacement of the acyl side chain at the *sn*-1 position with alkyl or ether side chains gives compounds that are still hydrolyzed by PLC_{Bc}, whereas such substitutions at the *sn*-2 position are not tolerated; removal of *sn*-2 carbonyl group completely abolishes recognition by PLC_{Bc}.[8] This fact may be rationalized by examination of the crystal structure of PLC_{Bc} in a complex with a substrate analog inhibitor.[9] In this structure, the carbonyl oxygen of the *sn*-2 ester forms a hydrogen bonding interaction with the backbone nitrogen of Asn134.

Substitutions that increase the distance between the phosphate and the nitrogen of the headgroup also lead to compounds that are not processed by PLC_{Bc}.[8] Although replacement of the choline headgroup with a *p*-nitrophenol ring afforded a compound that was not a substrate for the enzyme,[10] PLC_{Bc} does recognize and process compounds with more conservative headgroup modifications. For example, dihexanoyl phosphatidylethanolamine and dihexanoyl phosphatidylserine are cleaved by PLC_{Bc} at rates of approximately 0.5 and 0.25 fold, respectively, that of dihexanoyl phosphatidylcholine.[11]

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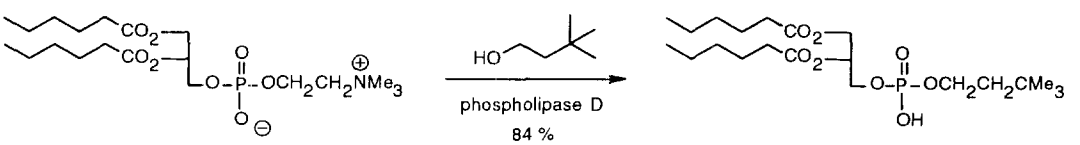
We have recently undertaken a program aimed at elucidating the catalytic mechanism of PLC_{Bc}. Towards this goal, we have prepared and evaluated a number of site-directed mutants of the enzyme.[6] One challenge is to determine what factors cause the differential processing of phospholipids bearing various headgroups by PLC_{Bc}. From crystallographic data, the choline binding region of the active site of PLC_{Bc} appears to consist of Glu4, Tyr56, and Phe66. The side chain of the glutamic acid presumably stabilizes the positively charged choline group via an electrostatic interaction, whereas the two aromatic residues are thought to be involved in a π-cation interaction with the trimethylamine moiety, a process that is just beginning to be recognized as vital in a number of biological systems.[12,13] The roles of these residues in substrate recognition by PLC_{Bc} has been confirmed by site-directed mutagenesis studies, the results of which indicate a large loss of activity upon the non-conservative replacement of the 4, 56, and 66 side chains.[14]

To begin to understand choline binding by PLC_{Bc}, the importance of the positive charge in the natural phosphatidylcholine substrate must be assessed. Synthesis of a compound with a *tert*-butyl group in place of the trimethylamine moiety would provide a phospholipid having identical steric requirements as the natural substrate, but lacking the positive charge. Because traditional phospholipid syntheses are multistep processes we searched for a method that would provide quick access to this target compound. This report describes the use of phospholipase D in a transphosphatidylation reaction with **1** to provide compound **2** (Scheme 1) and the subsequent kinetic evaluation of this novel *tert*-butyl phospholipid.

Scheme 1



1 **2**

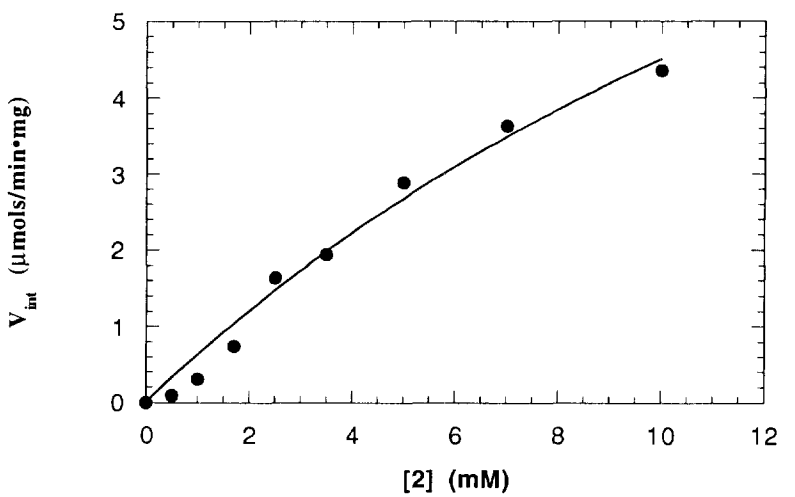
Phospholipase D (PLD) from *Streptomyces* species has been used in transphosphatidylation reactions with alcohols in the synthesis of phospholipids with unnatural headgroups.[15-18] Secondary[19] and aromatic [20,21] alcohols have been successfully incorporated via this enzymatic reaction. In the extension of this methodology to create **2**, PLD (Sigma P-4912) and 6 equiv of 3,3-dimethyl-1-butanol in 0.1 M NaOAc, 0.05 M $CaCl_2$ (pH 6.5, 0.2 mL), were added to dihexanoyl-*sn*-phosphatidylcholine (**1**) in $CHCl_3$ (1.7 mL) and incubated with shaking at 30 °C for 4.5 h. Extractive aqueous workup with $CHCl_3$ followed by flash chromatography on silica gel ($CHCl_3$:MeOH:H_2O/8:1.8:0.2 + 3% triethylamine) afforded a 84% yield of **2** as a colorless oil.[22] If triethylamine was not present in the eluant, the product decomposed on the column.

With compound **2** in hand, it was evaluated as a substrate for PLC_{Bc}. The assay was performed via a highly sensitive method based on the quantitation of inorganic phosphate.[11] In this assay, the phosphorylated headgroup produced is treated with alkaline phosphatase to generate inorganic phosphate. This inorganic phosphate then forms a complex with ammonium molybdate, which is reduced to the molybdenum blue state by

ascorbic acid. The resulting blue color is read at 700 nm by a microplate reader. The results of the assay with PLC_{Bc} and **2** are displayed graphically in Figure 1. A nonlinear least squares fit of the data utilizing the program KaleidaGraph gave a V_{max} = 11 μmols/min•mg, k_{cat} = 5.2 sec^{-1}, and a K_m = 14 mM. Previously substrate **1** had been found to exhibit a k_{cat} = 1000 sec^{-1} and a K_m = 2.4 mM.[11] Therefore, there is an approximately 1000-fold decrease in catalytic efficiency (k_{cat}/K_m) for **2** relative to **1**.

PLC_{Bc} is known to have a greater activity on some substrates when they are in their micellular form. For example, the activity of PLC_{Bc} on **1** increases roughly three-fold above the critical micelle concentration (CMC), whereas the activity of PLC_{Bc} on the corresponding phosphatidylserine analogue shows no large jump above the CMC.[11] The CMC of **2** was determined to be 9.5 mM using a dye inclusion assay,[23] but kinetic assays conducted at 14 mM of **2**, which is well above its CMC, showed no unusual increase in catalytic activity (data not shown).

Figure 1



The kinetic data for **2** demonstrates the relative importance of the positively charged nitrogen in phosphatidylcholine as a key recognition element for PLC_{Bc}. Removal of this charge results in a substrate that is processed ca. 1000 less efficiently than **1**. Although the primary effect is upon k_{cat} (200 fold decrease), there is also an approximately five-fold increase in the K_m. Experiments to quantitatively evaluate the magnitude of the specific interactions of different phospholipid headgroups with the choline binding pocket of PLC_{Bc} that is defined by Glu4, Tyr56, and Phe66 are underway, and these results will be presented in due course.

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22. Spectral properties of **2**. ^{1}H NMR δ 5.22-5.19 (m, 1H), 4.34 (dd, J = 3.9, 11.8 Hz, 1 H), 4.15 (dd, J = 6.4 Hz, 1 H), 4.07 (dd, J = 6.4 Hz, 1 H), 4.02 (dd, J = 4.02 Hz, 1 H), 3.08 (t, J = 7.3 Hz, 1 H), 3.06 (t, J = 7.3 Hz, 1 H), 2.30 (t, J = 7.5 Hz, 2 H), 2.28 (t, J = 7.3 Hz, 2 H), 1.59 (t, J = 7.5 Hz, 4 H), 1.35 (t, J = 7.3 Hz, 2 H) 1.28-1.22 (m, 8 H), 0.91 (s, 9 H), 0.87 (t, J = 6.8 Hz, 6 H); ^{13}C NMR δ 173.3, 172.9, 69.9, 63.9, 62.3, 45.6, 34.1, 34.0, 31.2, 29.6, 27.7, 24.5, 22.3, 16.2, 13.9, 8.5; ^{31}P NMR δ 0.05 (s); IR (film) ν 3438, 2959, 2872, 1739, 1644, 1470 cm^{-1}; mass spectrum *m/z* 453.2614 ($C_{21}H_{42}O_8P$ requires 453.2617).
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