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Benzene-di-*N*-octylcarbamates as conformationally constrained phospholipase A_2 inhibitors

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Abstract—Conformationally constrained 1,2-, 1,3-, and 1,4-benzene-di-*N*-octylcarbamates are potent reversible competitive inhibitors of *Naja mocambique mocambique* phospholipase A_2 with the K_i values of 11, 4, and 15 μM, respectively. With the angle of 120° between two C(benzene)–O bonds, 1,3-benzene-di-*N*-octylcarbamate mimics the preferable eclipsed C(*sn*-2)–O/C(*sn*-3)–O conformer of phospholipid in the enzyme-phospholipid complex. Further, a three-step phospholipase A_2 inhibition mechanism by the inhibitor is proposed.

Phospholipase A_2 (PLA2, EC 3.1.1.4) catalyzes hydrolysis of the *sn*-2 ester bonds of phospholipids, liberating lysophospholipid and free fatty acid products. Apart from being the products of phospholipid digestion, fatty acids and lysophopholipids may be transformed into inflammatory lipid mediators, thereby implicating PLA2s in the pathogenesis of many inflammatory disease states. In fact, PLA2s are found in variety of extracellular locations and are involved in a range of physicological processes including phospholipid digestion, signal transduction, and host defense.[1] PLA2 has served as useful prototype for elucidating the mechanism for an enzyme that functions at a lipid–aqueous interface.[2]

The X-ray crystal structure of a complex between a transition state analogue, 1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine and *Naja naja atra* PLA2 has shown that the active site Ca^{2+} binds to both the *sn*-2 phosphonate oxygen and the *sn*-3 phosphodiester oxygen of the inhibitor and that the dihedral angle between the C(*sn*-2)–O and C(*sn*-3)–O bonds is about 180° (*anti* conformation).[3] However, the X-ray structure of the phosphonate inhibitor–porcine PLA2 complex has revealed the same the active site Ca^{2+} binding to those oxygens but the dihedral angle is about 60° (*gauche* conformation).[4] Thus, the glycerol *sn*-2–*sn*-3 backbond conformation may play an important role in the PLA2 catalysis. We therefore design 1,2-, 1,3-, and 1,4-benzene-di-*N*-alkylcarbamates (Fig. 1) as the conformationally constrained analogues of glycerides, such as triglycerides and phosphatidylcholines (Fig. 2) because the angle between two C (benzene)–O bonds mimics the dihedral angle between two adjacent glycerol C–O bonds of glycerides (Fig. 2). In other words, 1,2-, 1,3-, and 1,4-benzene-di-*N*-alkylcarbamates may mimic the *gauche*-, *eclipsed*-, and *anti*-conformers for the C(*sn*-2)–C(*sn*-3) backbone of phosphatidylcholine, respectively (Fig. 2).

Among 30 carbamate compounds (Fig. 1), only three di-octylcarbamates (**1**–**3**) are competitive inhibitors of *Naja mocambique mocambique* PLA2 (Table 1). Thus, PLA2 selectively reacts with the inhibitors with two octycarbamate substituents; however, the other carbamate compounds are too hydrophilic to enter the enzyme active site through the hydrophobic i-face of the enzyme.[2] Among carbamates **1**–**3**, *meta* carbamate **2** is the most potent inhibitor of the enzyme (Table 1). Therefore, the *sn*-2–*sn*-3 backbone conformation of phosphatidylcholine that binds to the enzyme active site may adapt the *eclipsed* conformation (Fig. 2).

The PLA2 inhibition mechanism by carbamates **1**–**3** is proposed (Fig. 3). The first step (K_b) is protonation of carbamates **1**–**3** since carbamates **1**–**3** ($pK_a = 9 \pm 1$) are basic in aqueous solution.[5] The second step (K_S) is formation of the octa-coordinated Ca^{2+}-PLA2-inhibitor

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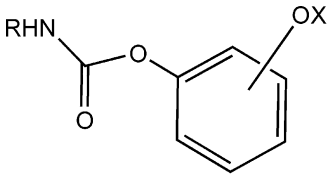

R = Bu, Hex, Oct, t-Bu, Bn;
X= H, CONHR

Figure 1. Structures of 1,2-, 1,3-, and 1,4-benzene carbamates.

complex by insertion of one of two octylcarbamate groups of protonated carbamates **1**–**3** to the hepta-coordinated Ca^{2+} of the native PLA2.[2,6] The third step (k_2/k_{-2}) is the chemical step for formation of the enzyme-inhibitor tetrahedral intermediate from a nucleophilic attack of the equatorial water-5 that is activated by the nearby water-6 which is deprotonated by the His49-Asp99 catalytic diad (calcium-coordinated oxyanion mechanism).[2,6] The dissociation constant for the octa-coordinated Ca^{2+}-PLA2-inhibitor complex, $K_S = K_i k_2/k_{-2}$, can be obtained from eq 1, where k_{obs}, k_2, and k_{-2} are the pre-steady state first order rate constant, the formation rate constant for the enzyme-inhibitor tetrahedral intermediate, and the re-dissociation rate constant for the enzyme-inhibitor tetrahedral intermediate, respectively.[7–14]

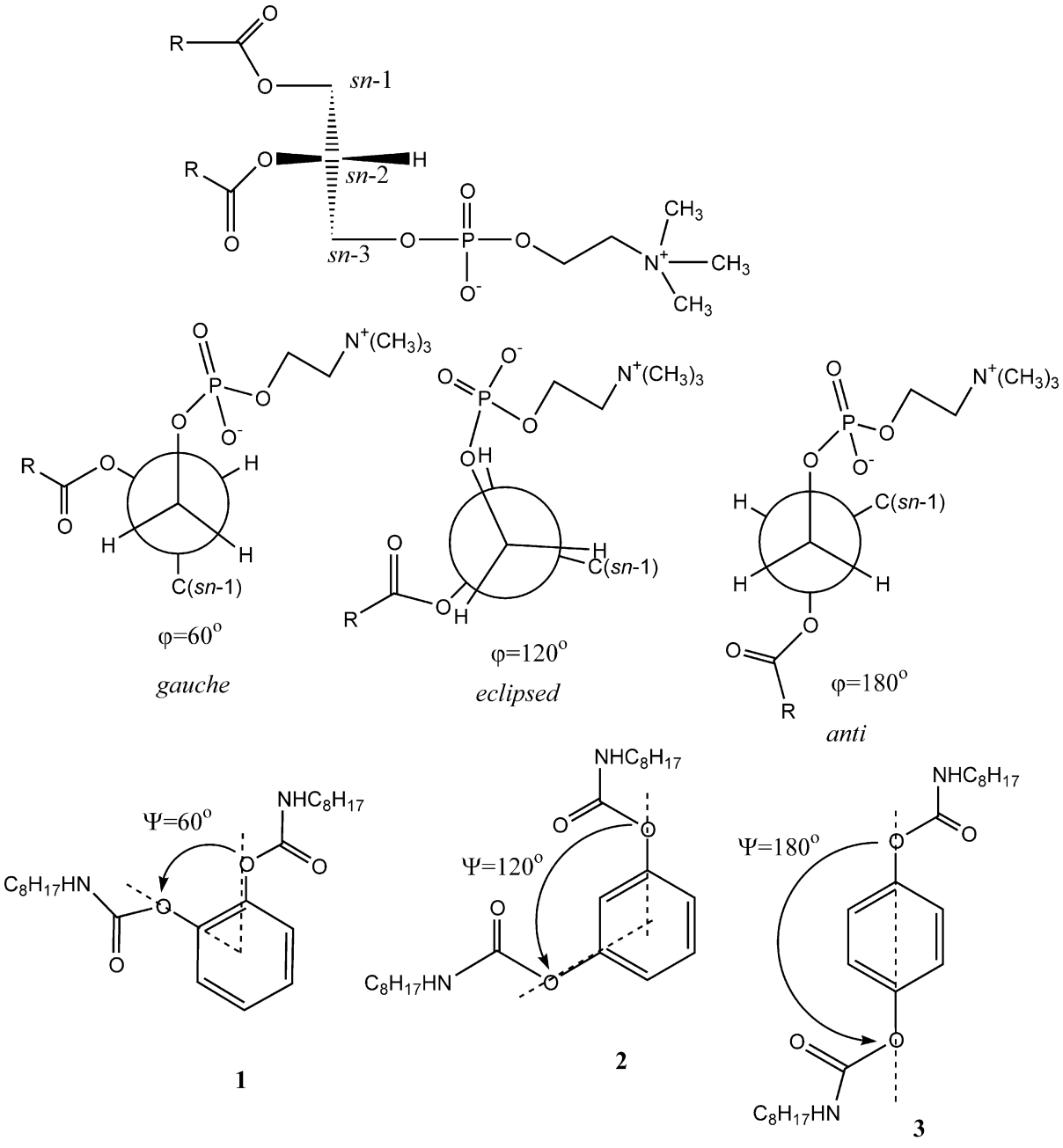

Figure 2. Three conformers of phosphatidylcholines and structures of carbamates **1**–**3**.

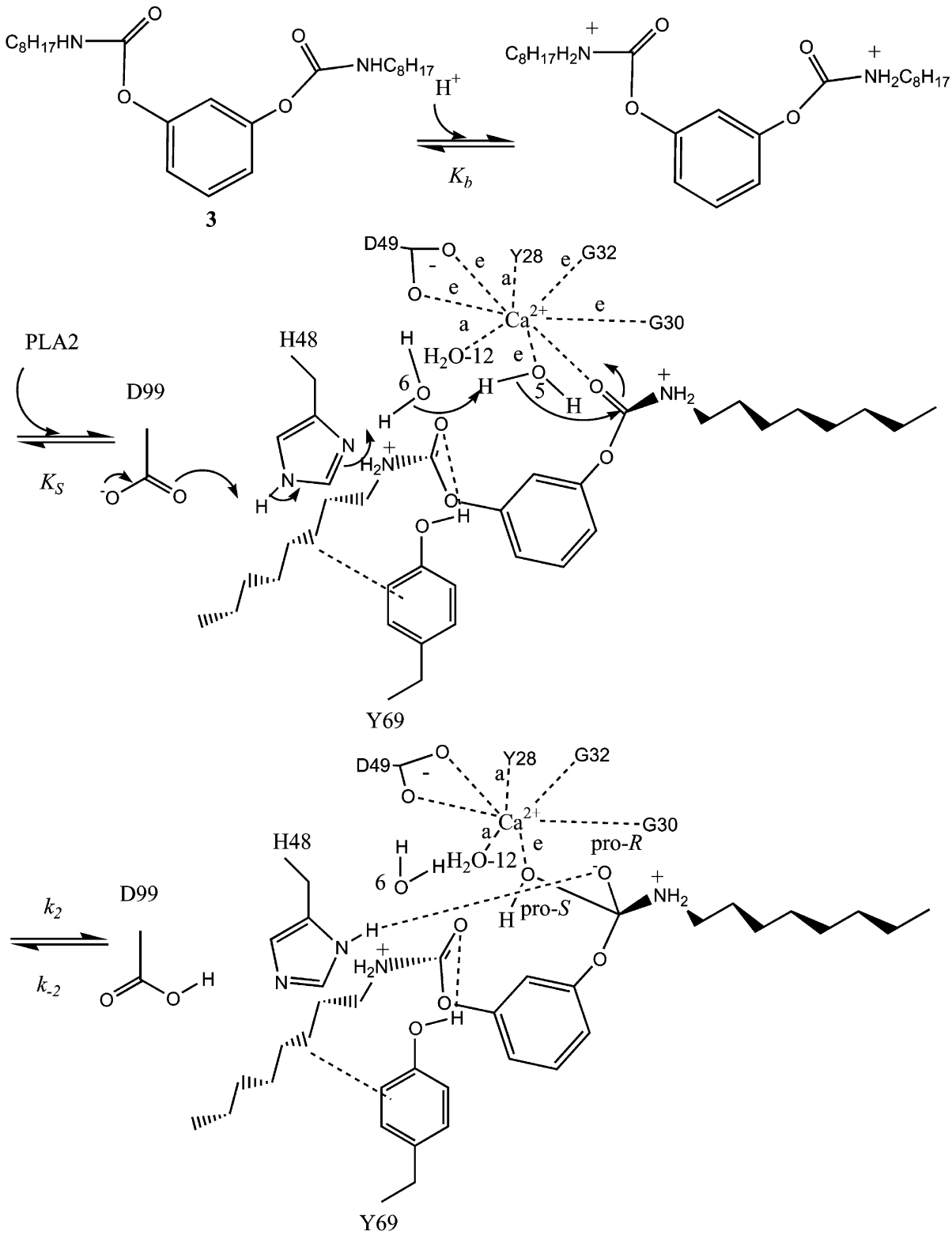

Figure 3. The proposed PLA2 inhibition mechanism by carbamate **2**. The first step (K_b) is protonation of carbamate **2**. The second step (K_S) is insertion of one of two octylcarbamate groups of protonated carbamate **2** into the hepta-coordinated Ca^{2+} of the enzyme.[6] The third step (k_2/k_{-2}) is formation of the enzyme-inhibitor tetrahedral intermediate via a nucleophilic attack of the equatorial water-5 that becomes the pro-*S* oxygen of the tetrahedral intermediate and is deprotonated and activated by the nearby water-6 (calcium-coordinated oxyanion mechanism).[6] Water-6 is activated by the His48-Asp99 diad. The pro-*R* oxygen of the tetrahedral intermediate binds to His48. A replacement of the apical water-12 by the other octylcarbamate group in this tetrahedral intermediate does not occur.[6]

$$k_{obs} = k_{-2} + k_2\,[I]/(K_S + [I]) \quad (1)$$

The K_S value for *ortho* carbamate **1** is the greatest one (Table 1) probably because one of two octylcarbamate substituents of the inhibitor lessens the other *ortho* octylcarbamate substituent of the inhibitor to insertion into the hepta-coordinated Ca^{+2} of PLA2[2,6] (Fig. 4). *para* carbamate **3** is the least potent inhibitor due to the largest k_{-2} value (Table 1). This is probably because free rotations of two *para* C(benzene)–O bonds of the inhibitor instabilize the hydrogen bond between His48 of the enzyme and *pro*-R oxygen of the inhibitor as well as the enzyme-inhibitor tetrahedral intermediate (Fig. 5).

Carbamates **1**–**3** also inhibit mammalian porcine pancreatic PLA2 (Fig. 6) and therefore are potential candidates for non-steroidal anti-inflammatory drugs (NSAIDs). Further investigations for the inhibitions of pancreatic and bee venom PLA2s by carbamates **1**–**3** will be communicated in due course.

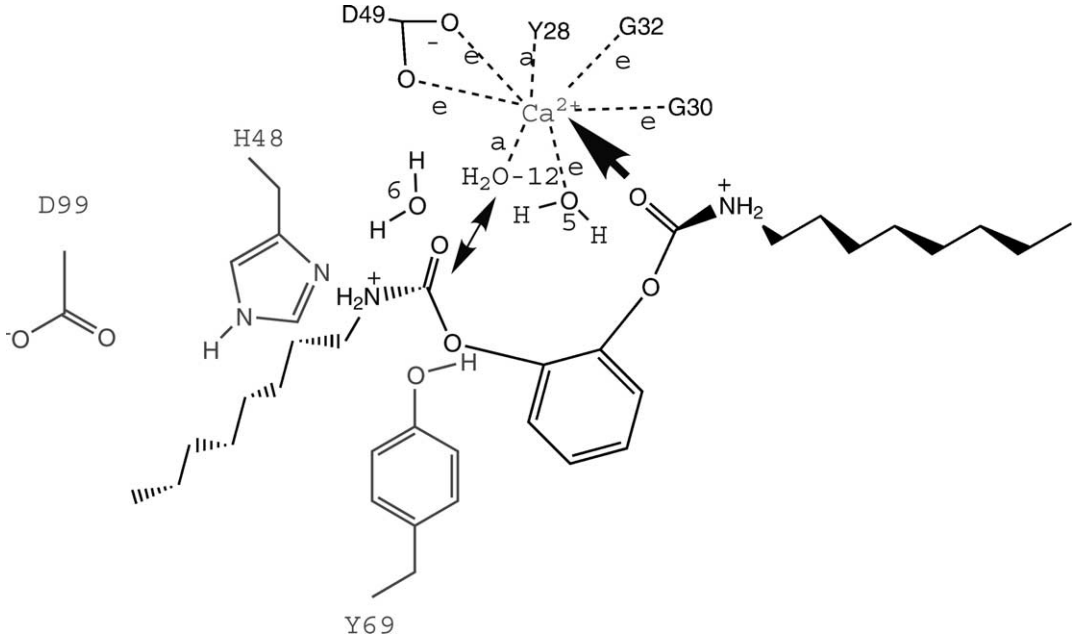

Figure 4. Insertion of protonated carbamate **1** to PLA2. The insertion of one of two octylcarbamate groups of protonated carbamate **1** into the hepta-coordinated Ca^{2+} of the enzyme is inhibited by the other octylcarbamate group of the inhibitor.

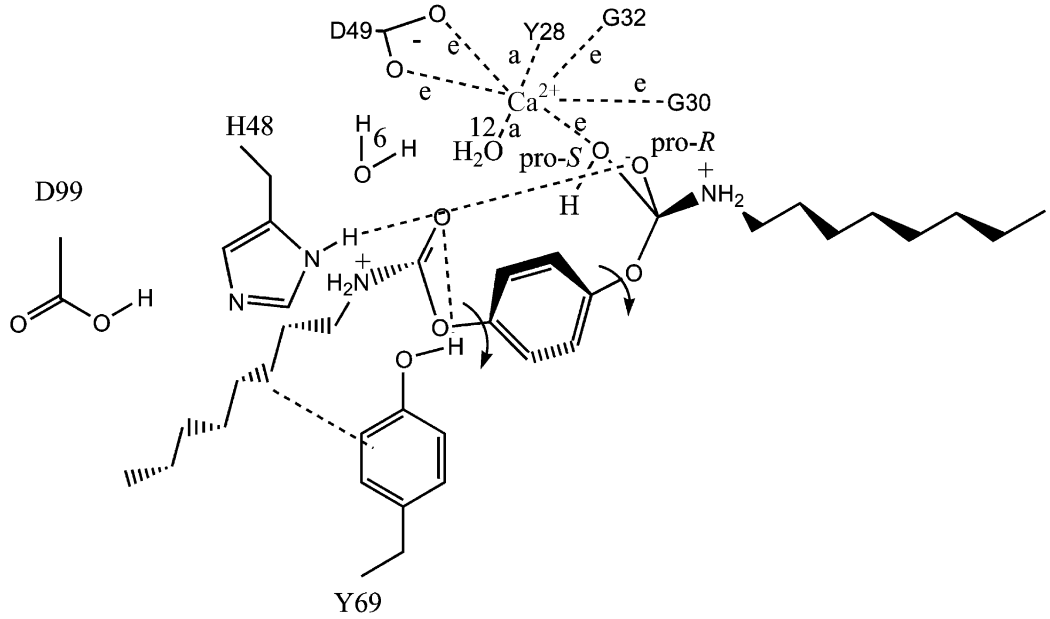

Figure 5. Interactions in the PLA2-protonated carbamate **3** tetrahedral intermediate. The hydrogen bond between the pro-*R* oxygen of the enzyme-protonated carbamate **3** tetrahedral intermediate and His48 of the enzyme is weakened by free rotations of two *para* C(benzene)–O bonds.

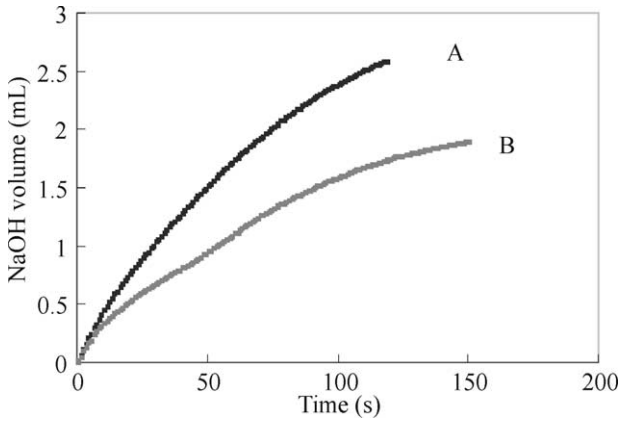

Figure 6. Porcine pancreatic PLA2 inhibition by carbamate **3**. Porcine pancreatic PLA2 (Sigma)-catalyzed hydrolysis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (0.1 mM) with (curve B) or without (curve A) carbamates **3** (0.8 mM).

Table 1. Steady- and pre-steady-state inhibition constants of PLA2 inhibitions by carbamates **1–3**

Inhibitors[a]	K_i (μM)[b]	K_S (μM)[c]	k_2 (s^{-1})[c]	k_{-2} (s^{-1})[d]
1	11±1	68±5	0.17±0.02	0.028±0.005
2	4.0±0.7	16±2	0.13±0.01	0.033±0.008
3	15±2	21±2	0.21±0.01	0.15±0.03

[a] Carbamates **1–3** were prepared from condensation of 1,2-, 1,3-, or 1,4-benzene-diol with 2.5 equivalents of octylisocyanate in triethylamine at 25 °C for 24 h (80–90% yield).
[b] The K_i values were obtained from the Lineweaver–Burk plots. Steady-state initial rates of *Naja mocambique mocambique* PLA2 (Sigma)-catalyzed hydrolysis of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Sigma, 0.1 mM) in the presence of carbamates **1–3** were followed by pH-stat titration (Radiometer PHM 290) under a stream of nitrogen at pH 8.0 and 25 °C in a 5-mL reaction mixture containing NaCl (10 mM).
[c] Pre-steady state inhibition rate constants were calculated from nonlinear least squares regression of eq 1 for the initial burst phase of the pH-stat titration.
[d] Calculated from $1/K_i = (1/K_S)(k_2/k_{-2})$.

Acknowledgements

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