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Efficient Synthesis of 3-Trifluoromethylphenyldiazirinyl Oleic Acid Derivatives and Their Biological Activity for Protein Kinase C

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Abstract—3-Trifluoromethylphenyldiazirine based oleic acids derivatives are synthesized to elucidate the functions of specific activation of protein kinase C (PKC) with oleic acid. The synthetic route is based on the alkylation of phenolic derivative with oleic acid equivalent and the post-functionalization of the compound to achieve radiolabeling. Several compounds have biological activity for PKC with similar efficacy with that of oleic acid. The results indicated that the diaizinyl oleic acid derivatives should be useful to study the specific functions of oleic acid for PKC.

Protein kinase C (PKC) plays a pivotal role in transmembrane signaling in response to various hormones and neurotransmitters.[1,2] It has been established that PKC is regulated by diacylglycerol (DAG) generated by receptor mediated phospholipid hydrolysis. The binding site of DAG in PKC has been determined using tumor promoting phorbol esters that bind to PKC with high affinity in a competitive manner with DAG.[3] In addition to DAG, PKC can be activated by free unsaturated fatty acid such as arachidonic acid and oleic acid.[1] However, the binding site of free fatty acid within PKC has not been determined. This is partly because PKC requires relatively high concentrations of free fatty acid for its activation, and there is no agent available that mimics free fatty acid with high affinity binding to PKC. Recently, it was demonstrated that PKC translocation occurs at remarkably low concentrations of arachidonic acid; a brief exposure of low concentrations (10–30 nM) of arachidonic acid translocates PKC from the cytosol to the membrane.[4] Other free unsaturated fatty acids have the similar effect on PKC translocation but arachidonic acid metabolites, such as leukotriene B4 and 5-hydroxyicosatetraenoate (5-HETE), do not have such an effect. This indicates that free fatty acid itself, but not arachidonic acid metabolites, mediates the translocation of PKC. Furthermore, arachidonic acid at similar low concentrations stimulated the binding of PKC to tumor promoting phorbol esters.[4] These findings that free unsaturated fatty acid is able to bind to PKC with high affinity and translocate it in vivo at low concentrations prompted us to develop photoreactive fatty acid derivatives for studying the fatty acid regulation of PKC photoaffinity labeling in vivo. Oleic acid was chosen over arachidonic acid since oleic acid is not metabolized through cyclooxygenase/lipoxygenase pathways and therefore the effect on PKC can be directly attributed to fatty acid itself but not to its metabolites.

Photoaffinity labeling is a powerful method in the study of the structure and function of biomolecules.[5–10] The technique has been widely used for analysis of interactions in vivo based on the affinity of the ligand moiety with its target macromolecules. Various photophors such as phenyldiazirine, arylazide and benzophenone, have been used. Comparative irradiation studies of these photophors for living cells suggested that a carbene precursor 3-trifluoromethylphenyldiazirine is most promising.[11] However, the complicated synthesis of the 3-trifluoromethylphenyldiazirinyl three membered ring has resulted in fewer applications of it in biomolecular studies than those of other photophors. The synthesis of

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photoreactive oleic acid analogues presents a similar problem. Ruhmann and Wentrup has been reported diazirinyl oleic acid synthesis.[12] But the synthesis consist of over ten steps, because the oleic acid equivalents should be introduced before construction of diazirinyl three membered ring. Furthermore, there is no report for the biological activity for the diazirinyl oleic acid derivative. We have been elucidating approaches for the post-functionalization of the 3-trifluoromethylphenyldiazirinyl photophor.[13–18] In the course, we found the diazirinylphenoxy linked saturated fatty acid derivatives was recognized as substrate by biomolecules.[15] In this paper, we describe effective synthesis of 3-trifluoromethylphenyldiazirine based oleic acid analogues and their substituent effects for PKC activity.

Figure 1 shows the synthesis of diazirine-based oleic acid derivatives. *m*-Hydroxydiazirine **1**[16] was subjected to the phenoxy alkylation condition[19] with 18-bromooleic acid methyl ester[20] to afford **4** with moderate yield. Then the methyl ester was hydrolyzed to yield oleic acid derivative **5**.[21] To investigate the effects of substituent groups on benzene ring for PKC activity, the compound **5** was subjected Friedel–Craft's reaction with dichloromethyl methyl ether[13] and acetyl chloride[18] to yield compound **6** and acetophenone derivative, respectively. But the double bond in the oleic acid was not stable under those conditions. To yield **6**, the 4-formyl-3-hydroxydiazirine **2**[22] was applied to phenoxy alkylation. The reaction proceeded without damage to aldehyde moiety. After ester hydrolysis, the aldehyde **7** was reduced to alcohol **8** with sodium borohydride, which is easy to purchase radiolabeled reagent. The compound **1** was subjected to iodination, which is one of the most common radioisotopes, with one equivalent of NaI and Chloramine T[23] to afford compound **3**. The monoiodinated compound also applied to phenoxy alkylation in a manner identical with above procedure. NOE measurement of *O*-linked methylene for compound **9** revealed the methylene correlated to only singlet benzene proton. The result indicated the orientation of iodine was confirmed to 4-position against diazirinyl moiety.

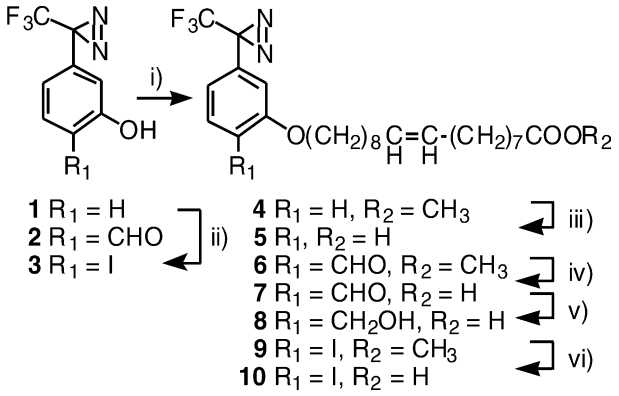

Figure 1. Synthesis of diazirine based oleic acid derivatives. (i) 18-Bromo oleic acid methyl ester, K_2CO_3, $(n\text{-}Bu)_4NI$, DMF, 60 °C, 58–60%, (ii) NaI, Chloramine T, CH_3OH, rt, 60%, (iii) NaOH, CH_3OH, rt, 2 h, 85%, (iv) NaOH, CH_3OH, rt, 71%, (v) $NaBH_4$, C_2H_5OH, rt, 90%, (vi) NaOH, CH_3OH, rt, 75%.

The biological activity of the synthetic compounds for the activation of PKC was tested using purified human recombinant PKCα (Pan Vera). As shown in Figure 2, all compounds, except compound **8**, have similar efficacy with oleic acid for PKC activation. The Km values are 102±21, 174±40, 186±63, and 140±99 μM for oleic acid, compound **5**, **7** and **10**, respectively. We have found that iodine substitution makes the compound slightly more potent for the activation of PKC at lower concentrations (Fig. 2). This result is consistent with the previous observation on the iodinated diazirinyl bisglucose makes slightly higher activity for glucose transporter than unsubstituted diazirinyl one.[24] Interestingly, the ability of benzyl alcohol type compound **8** to induce PKC activation was drastically reduced. This could be due to the direct interaction between the introduced alcohol moiety and PKC, although we do not know the precise nature of the interaction at the moment.

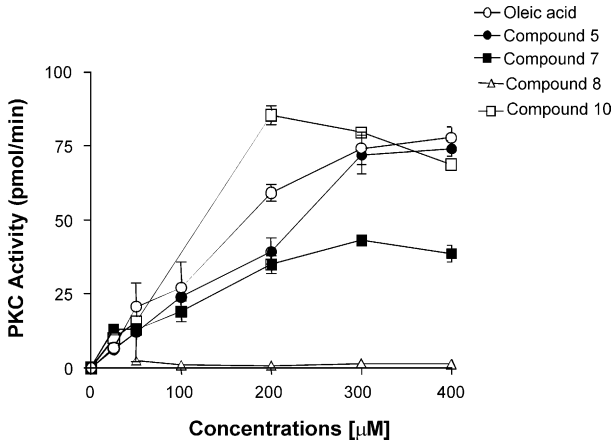

Figure 2. PKC activation by photoreactive oleic acid derivatives. Biological activity of the oleic acid derivatives were tested using lysine-rich histone as a phosphate acceptor substrate in the presence of 100 μM free Ca^{2+} using a method described previously.[25,26]

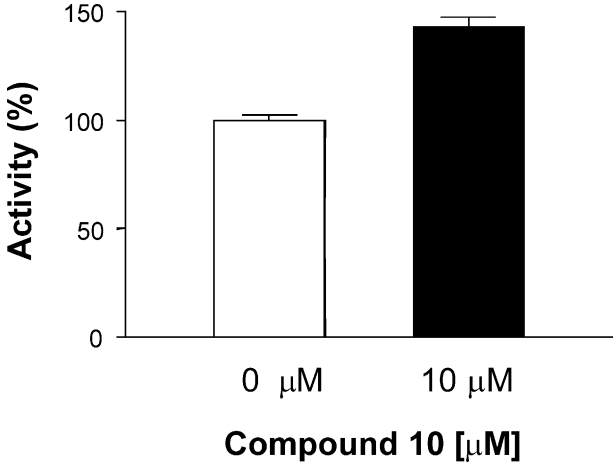

Figure 3. Effect of compound **10** at a low concentration on diacylglycerol-induced PKC activation. The PKC activity was measured in the absence or presence 10 μM compound **10** in the presence of 10 μM dioctanoyl-*sn*-glycerol and 10 μM dioleoylphosphatidylserine using lysine rich histone as a substrate.

We have examined the effect of compound **10** at low concentrations (1–10 μM) for DAG-induced PKC activation because oleic acid can activate PKC at much lower concentrations in the presence of DAG.[27] In this condition, compound **10** at 10 μM increases the DAG activity for PKC over 40% (Fig. 3). The result indicated photoreactive compounds have synergic effects with DAG as same as oleic acid.

The synthesis route of photoreactive oleic acid derivatives could be easily changed for the different carbon length of oleic acid equivalent and applied to synthesis of radiolabeled compounds without loss of bioactivity for PKC. These compounds should be useful to elucidate not only PKC activation but also other biological molecules that interact and are regulated by free unsaturated fatty acid.

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21. Compound **5** ^{1}H NMR ($CDCl_3$) δ 7.29 (1H, t, J = 8.2 Hz), 6.92 (1H, d, J = 8.2 Hz), 6.75 (1H, d, J = 8.2 Hz), 6.67 (1H, s), 5.35 (2H, m), 3.93 (2H, t, J = 6.6 Hz), 2.34 (2H, t, J = 7.6 Hz), 2.02 (4H, m), 1.77 (2H, m), 1.63 (4H, m), 1.46–1.26 (16H, m), compound **7** ^{1}H NMR ($CDCl_3$) δ 10.47 (1H, s), 7.84 (1H, d, J = 8.3 Hz), 6.82 (1H, d, J = 8.3 Hz), 6.68 (1H, s), 5.35 (2H, m), 4.06 (2H, t, J = 6.6 Hz), 2.34 (2H, t, J = 7.6 Hz), 2.02 (4H, m), 1.85 (2H, m), 1.60 (4H, m), 1.48–1.30 (16H, m), compound **8** ^{1}H NMR ($CDCl_3$) δ 7.31 (1H, d, J = 7.9 Hz), 6.78 (1H, d, J = 7.9 Hz), 6.61 (1H, s), 5.35 (2H, m), 4.69 (2H, s), 3.99 (2H, t, J = 6.6 Hz), 2.34 (2H, t, J = 7.6 Hz), 2.00 (4H, m), 1.81 (2H, m), 1.60 (4H, m), 1.43–1.25 (16H, m), compound **10** ^{1}H NMR ($CDCl_3$) δ 7.77 (1H, d, J = 8.2 Hz), 6.54 (1H, d, J = 8.2 Hz), 6.50 (1H, s), 5.35 (2H, m), 3.98 (2H, t, J = 6.3 Hz), 2.35 (2H, t, J = 7.6 Hz), 2.02 (4H, m), 1.83 (2H, m), 1.63 (4H, m), 1.52–1.31 (16H, m).
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