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STEREOSPECIFIC PROTEIN KINASE C ACTIVATION BY PHOTOLABILE DIGLYCERIDES

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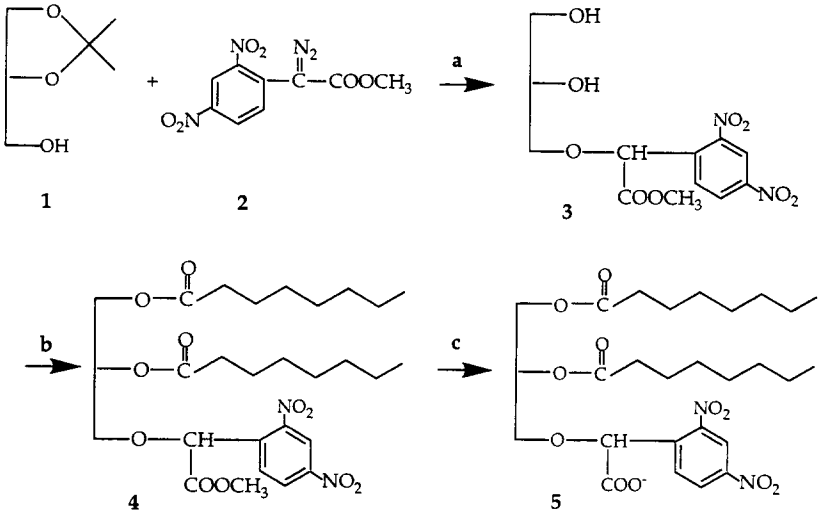
Abstract: The synthesis and photochemistry of diglycerides designed to photolyze to bioactive diacylglycerols and optimized for facile incorporation into biological membranes is described. Stereospecific activation of protein kinase C in vitro and in living cells is demonstrated.

Protein kinase C (PKC) is a ubiquitous family of enzymes that regulate many cellular proteins by phosphorylation.[1] PKC is inactive in the cytoplasm of cells until its primary activator, diacylglycerol, is produced by the enzyme phospholipase C.[1] Further understanding of the mechanism of diacylglycerol action and the role of PKC in specific cellular processes depends on the development of reagents that permit its concentration and localization to be precisely controlled. A powerful approach for controlled release of biological molecules including neurotransmitters, nucleotides, phosphates, and inorganic ions has been developed around light activation of photosensitive precursors.[2]

Here we report the synthesis, preliminary photochemical characterization, and biological properties of 3-O-(α-carboxyl-2,4-dinitrobenzyl) 1,2-dioctanoyl-rac-glycerol, termed α-carboxyl caged diC_8. This compound is a novel near-UV light activated ('caged') precursor of dioctanoylglycerol (diC_8). We chose diC_8 for our prototype compound because it is a known activator of PKC and possesses short acyl chains, which in combination with a negative charge on the photolabile moiety, facilitated incorporation of this class of compounds into cell membranes.[3] We also resolved the racemic mixture of synthetic caged diC_8 into R and S isomers, and characterized the biological properties of each.

Chemicals for synthesis were obtained from Aldrich (Milwaukee, WI), lipids were from Avanti Polar Lipids (Alabaster, AL), and biochemicals were from Sigma Chemical Co (St. Louis, MO). HPLC was performed with a pair of Beckman 110B pumps, a 210A sample injector, and a 160 absorbance detector at 254 nm. α and ε isoforms of PKC were purified from cultured insect cells by standard procedures.[4] The synthetic route for large scale (≥100 mg) synthesis of α-carboxyl caged diC_8 is shown in Scheme 1. Diazo[2,4-dinitrophenyl] acetic acid methyl ester (**2**): Synthesis was carried out by the reported procedure[5] and purified by flash chromatography in hexane:ethylacetate.[6] α-Carboxyl caged glycerol methyl ester (**3**): Compound **2** (0.38 mmol, 100 mg) was dissolved in 0.94 mL (7.6 mmol, 1.0 g) solketal (**1**) by heating at 40-50 °C with bath sonication. A few drops of BF_3-etherate were added and sonication continued for an additional 30 min. The isopropylidene protecting group was removed by treatment with 50% glacial acetic acid for 3 h. Compound **3** was purified by semipreparative HPLC using 30% acetonitrile in water at 5 mL/min as an oil (55% yield).[7]



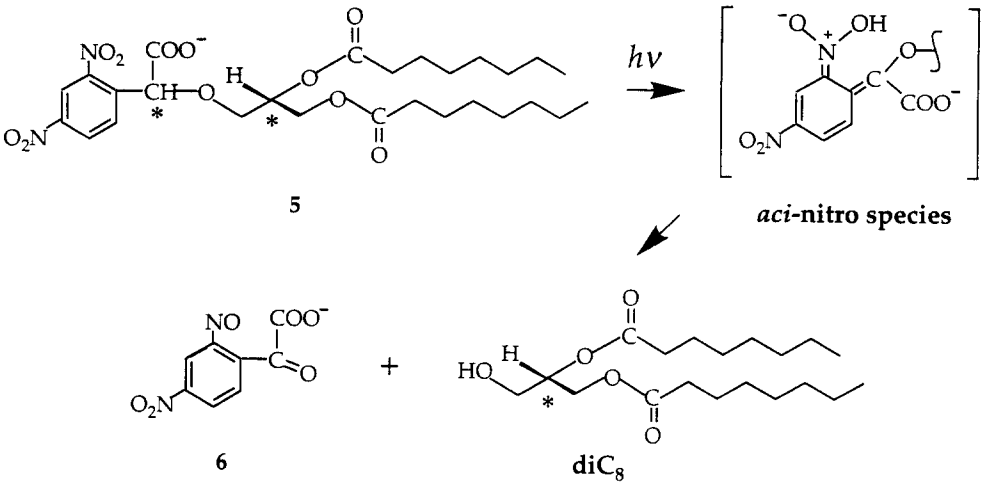
Scheme 1. (a) i. BF_3-etherate ii. 50% HOAc; (b) $C_8H_{15}OCl$, pyridine; (c) pyridine/LiI

α-Carboxyl caged dioctanoylglycerol methyl ester (**4**): Octanoyl chloride (3 mmol, 490 mg) was added to a stirred solution of **3** (1 mmol, 330 mg) in 3 mL CH_2Cl_2 and 0.1 mL dry pyridine. After 30 min, the reaction mixture was washed with 3% H_2SO_4 and extracted with CH_2Cl_2. Removal of CH_2Cl_2 furnished the crude product, which was purified by column chromatography using 20:1 hexane:ethylacetate (40% yield).[8] Compound **4** is stable for at least 6 months at -80 °C.

3-O-(α-carboxyl 2,4-dinitrobenzyl)-1,2-dioctanoyl-rac-glycerol (α-carboxyl caged diC_8) (**5**): Compound **4** (10 μmol, 5.7 mg) in CH_2Cl_2 was added to a siliconized plastic vial and the solvent was removed by a N_2 stream. 500 μL of 100 mg/mL LiI in anhydrous pyridine was added under N_2 and the reaction proceeded at room temperature over 4-5 h. α-Carboxyl caged diC_8 (**5**) was purified by semipreparative reverse-phase HPLC using 70% acetonitrile in water at 5 mL/min as an oil (45% yield).[9] Compound **5** is stable for approximately 1 month at -80 °C and is typically prepared from **4** as needed.

The synthesis is general in that diglycerides containing other photolabile moieties such as the 2-nitrophenylethyl[10] or 4,5-dimethoxy-2-nitrobenzyl group[11] can be prepared starting with the appropriate diazonium compound. We had no success preparing the diazonuim derivative of methyl α-carboxyl-2-nitrobenzyl, so the 2,4 dinitro photolabile group was used here because it formed a stable diazonium and yet reacted readily with free alcohols. The α-carboxyl group was protected to prevent its esterification during this diazo coupling step. Diglycerides containing other acyl chains such as stearoyl, oleoyl and arachidonyl groups could be prepared on a large scale by acylation of **3** or on a small scale (0.1 - 10 mg) by coupling **2** directly with commercially available diglycerides. Compound **2** may be a general reagent for introducing the

the charged α-carboxyl 2,4 dinitro group into a variety of caged alcohols, phosphates and carboxylic acids.



Scheme 2. Proposed photolysis reaction for α-carboxyl caged diC_8.

Photochemistry

The presumed photolysis reaction for α-carboxyl caged diC_8 is illustrated in Scheme 2. In a previous report, we showed that near-UV irradiation of α-carboxyl caged diC_8 decreased the area of its HPLC peak concomitant with an increase in formation of biologically active diC_8.[12] Here we show that near UV-irradiation of **4** (the methyl ester of **5**) produces both biologically active diC_8 (Figure 1B) and a by-product (the methyl ester of **6**). Compound **4** was exposed to a 75 watt xenon lamp filtered at 300-400 nm (UG11 filter, Chroma Technologies, Inc) for 15 min in CH_2Cl_2. The methyl ester of **6** was purified by semipreparative reverse-phase HPLC in 50% acetonitrile and its structure confirmed by NMR, UV and mass spectra.[13] The ionized acid form of α-carboxyl caged diC_8 (**5**) produced a by-product with a similar UV-Vis spectrum to the methyl ester of **6** but was too unstable to isolate and further characterize. The identification of diC_8 and the ester of **6** as major products of photolysis of **4** provides support for the photolysis mechanism in Scheme 2 for the methyl ester and by inference for the ionized acid form of α-carboxyl caged diC_8.

Flash photolysis of **4** and **5** each revealed a transient intermediate in the photolysis reaction similar to that observed for other 2-nitrobenzyl compounds.[2,10] The metastable photolytic intermediate for 2-nitrobenzyl caged compounds has been assigned the structure of an *aci*-nitro species characterized by an absorbance maximum around 400 nm, and an exponential (in some cases bi-exponential[2]) decay process. The rate of this decay has been proposed as limiting the photochemical dark reaction and thus determines the rate of appearance of the biologically active photolysis product.[2,10] All photochemical properties described here were measured in 50% ethanol, 50 mM Tris pH 7.5, 22 °C. The 2,4-dinitro caged diC_8 compounds displayed a red shifted *aci*-

nitro intermediate (λ_{max} = 500 nm) suggesting extra resonance stabilization by the 4-nitro group, and decay rates of 2.5 s^{-1} (**4**) and 400 s^{-1} (**5**). Product quantum yields determined by comparison with caged phosphate[14] were 0.23 (**4**) and 0.18 (**5**). Thus, the 2,4-dinitro photolabile group photolyzes with good yield and rapidly enough to allow kinetic measurements to be made with caged diC_8, and may be of general applicability to other caged compounds.

Optical isomers of α-carboxyl caged diC_8

Synthetic caged diC_8 contains two asymmetric centers (shown in Scheme 2 by *), and thus four stereoisomers. Resolution of stereoisomers was achieved by chromatography of α-carboxyl caged diC_8 methyl ester on a chiral HPLC column (CHIRALPAK AD, 4.6 mm x 250 mm) (Figure 1A). Column eluant was monitored for optical rotation (ORD) and UV absorbance at 260 nm. Peaks 1 and 2 off the HPLC column showed no protein kinase C (PKC) activation before photolysis, but potently activated all PKC isoforms tested (α-PKC, ε-PKC, and mixed isoforms from rat brain) to greater than 85% of maximum after photolysis (Figure 1B). HPLC peaks 3 and 4 showed very little or no activation of PKC isoforms (< 15% of maximum) before and after photolysis. Thus, peaks 1 and 2 contain the active S-configuration in the glycerol backbone, and peaks 3 and 4 contain the inactive R-configuration. For comparison, racemic α-carboxyl caged diC_8 gave approximately 50% of maximum PKC activation after photolysis.

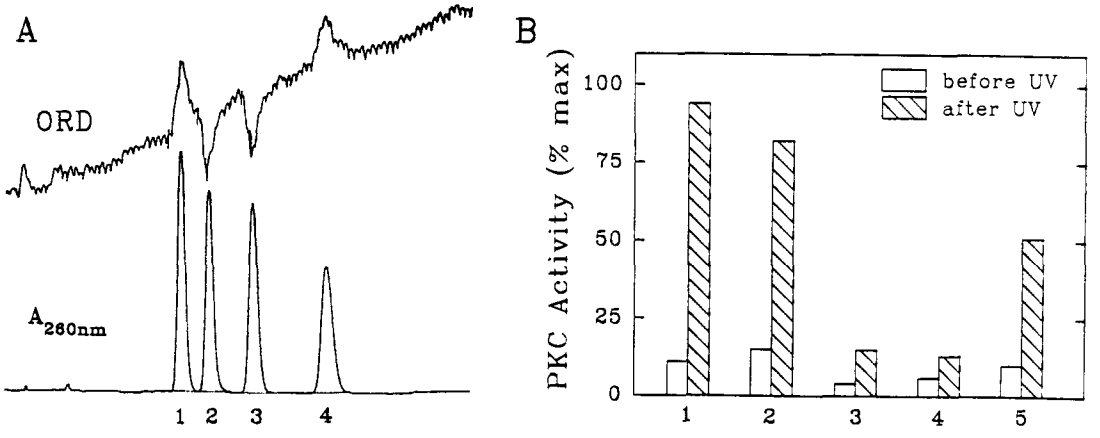


Figure 1. (A) Chiral HPLC separation of synthetic compound **4** into four isomers, 1-4, using hexane:ethanol (90:10) at 1 mL/min. (B) Activation of PKC by different stereoisomers and by racemic diC_8: 1 (HPLC peak 1), 2 (HPLC peak 2), 3 (HPLC peak 3), 4 (HPLC peak 4) and 5 (racemic compound **4**). Conditions: 50 μM caged compound in 50 mM Tris, 1 mM dithiothreitol, pH 7.5 was irradiated for 5 min in a 1 cm quartz cuvette and assayed for PKC activity.[4] ≤ 15% of maximal activity is considered background.

Cellular responses to photoreleased diC_8

The charged α-carboxyl group was found to be important for facile incorporation of caged diC_8 compounds into cell membranes. Compounds without the charged group such as α-carboxyl caged diC_8 methyl ester (**4**), 3-O-(2'-nitrobenzyl)-diC_8, 3-O-(2'-nitrophenylethyl)-diC_8, and 3-O-(4',5'-dimethoxy-2'-nitrobenzyl)-diC_8 were not incorporated into cardiac myocytes to a significant extent. We previously showed that continuous illumination of electrically stimulated cardiac myocytes that had been loaded with 250 μM α-carboxyl caged diC_8 (racemic) gave rise to a complex biological response.[12] Responses included an initial enhancement of cell contraction and an abrupt loss of responsiveness to electrical stimulation.[12] To address the possibility that some of this complexity is due to nonspecific effects of photolysis by-products or physical effects of diglyceride build-up within cell membranes, we examined responses to R and S isomers of α-carboxyl caged diC_8 (Figure 2). Both phases of the cellular response were observed following photorelease of (S)-diC_8, including a slowly developing enhancement of cell contraction and an abrupt loss of contractility (Figure 2B). In contrast, photorelease of (R)-diC_8 resulted in no change in cell contraction even at 4-fold higher concentrations (Figure 2A). Radiolabelled forms of (S)- and (R)-α-carboxyl caged diC_8 were incorporated into cardiac myocytes with equal efficiency, and steady-state illumination of samples prior to chiral HPLC separation caused a parallel decline of all four stereoisomers. Thus, differences in cellular responses to stereoisomers cannot be attributed to differences in incorporation efficiency or photosensitivity. Thus, both phases of the biological response to

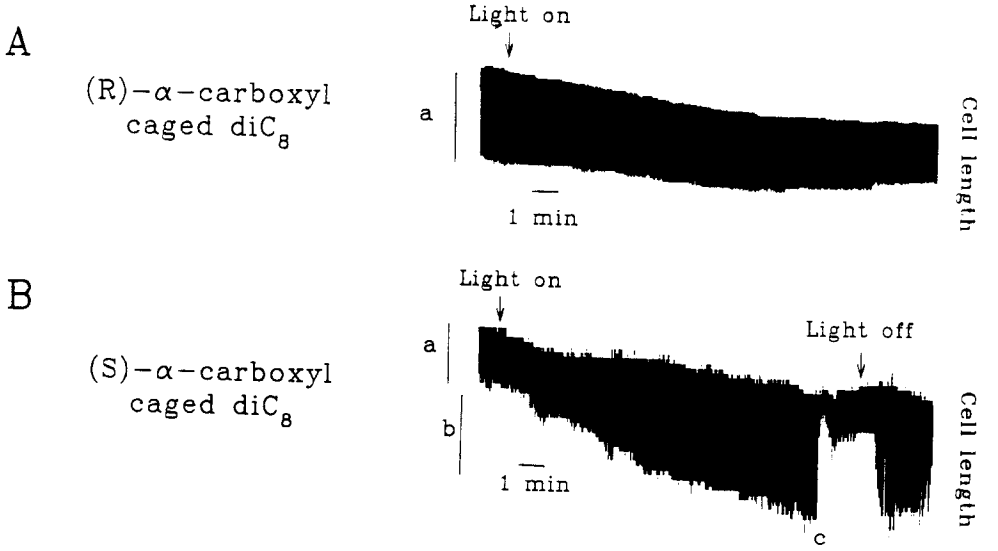


Figure 2. Stereospecific effects of photoreleased diC_8 on cardiac myocyte contraction. (A) Myocytes loaded with 1 mM (R)-caged diC_8 showed no change in basal contractility (a) in response to 15 min of illumination. (B) Myocytes loaded with 250 μM (S)-caged diC_8 showed both enhanced contractility (b) and a subsequent abrupt loss of contractility (c) in response to continuous illumination at 360 nm.

diC_8 were stereospecific for (S)-diC_8 and photolysis by-products did not detectably enhance or inhibit these cell responses. Combined with the observation that both phases were inhibited by preincubation with the PKC antagonist chelerythrine,[12] the results are consistent with biphasic effects of PKC activation on myocyte twitches.

In summary, α-carboxyl caged diC_8 is a new probe of PKC that is readily incorporated into cell membranes and is smoothly converted to biologically active diacylglycerol on illumination. Chiral HPLC was used to resolve stereoisomers of α-carboxyl caged diC_8 that had indistiguishable physical properties but displayed dramatically different efficacy towards PKC activation in vitro. These observations are consistent with an earlier report which showed that activation of PKC by diglycerides is stereospecific.[15] We extend this observation to specific isoforms of PKC (α and ε) and to the short chain analogue diC_8. In addition, we show that these stereoisomers can be used to elevate active or inactive diacylglycerols within living cells to evaluate the role of protein kinase C in complex cellular responses.

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6. 2: ^{1}H NMR ($CDCl_3$) δ 8.9 (s, 1H), 8.4 (d, 1H), 7.9 (d, 1H) and 3.85 (s, 3H). FABMS: m/z 266.02, calculated for $[C_9H_6O_6N_4^+]$ 266.17.
7. 3: ^{1}H NMR ($CDCl_3$) δ 8.85 (s, 1H), 8.63 (d, 1H), 8.28 (d, 1H), 5.82 (s, 1H), 4.13 (br s, 1H), 3.66-3.98 (m, 3H), 3.73 (s, 3H), 3.57 (d, 2H) 3.03 (br s, 1H). FABMS: m/z 331.0 (protonated molecular ion), calculated for $[C_{12}H_{14}O_9N_2^+]$ 330.23. UV-Vis (ethanol): λ_{max} 245 nm, ε_{245} 10,400 $M^{-1}cm^{-1}$.
8. 4: ^{1}H NMR ($CDCl_3$) δ 8.9 (s, 1H), 8.5 (d, 1H), 8.1 (d, 1H), 5.75 (s, 1H), 5.35 (m, 1H), 3.8-4.4 (m, 4H), 3.7 (s, 3H), 2.3 (t, 4H), 1.6 (m, 4H), 1.25 (m, 16H) and 0.85 (t, 6H). FABMS: m/z 583.2 (protonated molecular ion), calculated for $[C_{28}H_{42}O_{11}N_2^+]$ 582.62.
9. 5: ^{1}H NMR in d_6-DMSO of 5 was identical to 4 except the singlet at δ 3.7 was absent. FABMS: m/z 590.5 (sodium form of molecular ion), calculated for $[NaC_{27}H_{39}O_{11}N_2^+]$ 590.6.
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13. Methyl ester of 5: ^{1}H NMR ($CDCl_3$) δ 8.8 (s, 1H), 8.6 (d, 1H), 8.0 (d, 1H) and 3.8 (s, 3H). EI mass spectrum: m/z 238 (21), 149 (92), 103 (88) and 75 (89). UV-Vis (ethanol): λ_{max} 290 nm, ε_{290} 8,000 $M^{-1}cm^{-1}$; λ_{max} 750 nm, ε_{750} 50 $M^{-1}cm^{-1}$.
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