# Synthesis of Photoreactive Phosphatidic Acid Analogues Displaying Activatory Properties on Cyclic AMP-phosphodiesterases. Photoaffinity Labeling of an Isoform of Phosphodiesterase

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We have previously shown that phosphatidic acid (PA) is a specific activator of some isoforms of type 4 cyclic nucleotide phosphodiesterases (PDE 4) and that accumulation of endogenous PA can, in this way, influence the cAMP signaling pathway in different cell types. Enzyme activation depends on direct binding of the effector to specific sites carried by the enzyme. To identify the binding domain, photoactivatable phosphatidic acid analogues 1-azidoPA **(12)** and 2-azidoPA **(7** and **15)**, potentially suitable for covalent labeling of PDE4, have been synthesized. The ability of phospholipases $A_2$ and D to hydrolyze unnatural phospholipids has been considered in this paper. The effect of 1-azidoPA **(12)** and 2-azidoPA **(7** and **15)** on the activity of a recombinant PA-sensitive isoform PDE4D3 was evaluated. The three compounds were able to activate the enzyme with different efficiencies. A tritiated analogue of **15** was synthesized and used in PDE4D3 labeling experiments, which showed that this PA analogue was specifically and covalently linked to the enzyme after UV irradiation. Photoactivatable analogues thus appear as suitable tools for the characterization of PA binding sites.

## Introduction

Type 4 cAMP-phosphodiesterases (PDE4) constitute a family of enzymes characterized by their specificity for cAMP hydrolysis and their sensitivity to inhibition by the antidepressant compound rolipram. This PDE 4 family comprises numerous isoforms derived from the expression of four genes.[1] They have been shown to play a crucial role in the determination of cAMP levels in discrete cellular compartments, and as such, their activity modulates a variety of cell functions; they are, for example, involved in the control of differentiation of preadipocytes[2] and of skeletal myoblasts,[3] in the response of thyrocytes to thyrotropin,[4] and in reactive oxygen species production in mesangial cells.[5] A demonstration of the role of phosphodiesterase expressed from the gene PDE4D has been recently provided by gene inactivation studies in mice. The phenotype induced by PDE4D disruption includes growth retardation, decrease in female fertility, and loss of muscarinic airway responses in PDE4D null mice.[6,7] PDE4-specific inhibitors display interesting therapeutical potentials. Several compounds are undergoing clinical trials as antiinflammatory agents beneficial in the treatment of chronic obstructive pulmonary disease, rheumatoid arthritis,[8] and asthma.[9] We have previously shown that phosphatidic acid (PA) is a specific activator of some of the isoforms of PDE4.[10] This phospholipid is produced in many cell types in response to stimulation by hormones or growth factors and is thought to play a role as a second messenger. It is involved in cell functions such as vesicular traffic, respiratory burst,[11] and lymphocyte proliferation.[12] We have evidence that PDE4 activation by endogenously formed PA in the intact cell has a physiological relevance. Indeed, this regulation of PDE4 counteracts the thyrotropin-induced activation of the cAMP pathway in FRTL-5 thyroid cells,[13] and it is necessary for the proliferative response of peripheral blood lymphocytes.[14] Besides phosphodiesterases, the activity of several enzymes involved in signal transduction is modulated by PA, including protein phosphatase PTP1C,[15] raf-1 kinase,[16] etc. PA has been shown to specifically bind to some proteins (PKC $\zeta$,[17] PLC$\delta$3,[18] raf-1 kinase[16]), and a PA binding site has been characterized by genetic methods in the sequence of raf-1 kinase.[16] By studying the type 4 phosphodiesterase isoform PDE4D3, which is especially sensitive to PA activation, we have shown that radiolabeled PA specifically binds to the protein,[19,13] and a sequence including a PA binding site has been delimited by deletion mutation studies.[13] Covalent photoaffinity labeling of the enzyme with PA analogues seems to be a particularly appropriate approach to provide further support to the hypothesis that the PA-activating effect depends on direct binding of the effector to specific sites carried by the PDE4D3 protein. It might also help define the sequence of these PA binding sites. We now report the synthesis of photoreactive phosphatidic acid analogues and an evaluation of their biological activity as effectors of PDE4D3 recombinant enzyme showing that the three obtained compounds have affinity for the enzyme, which is a prerequisite for a successful affinity labeling. We also report the results of photolabeling experiments

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performed on PDE4D3, which show that a radioactive analogue of PA specifically labels the PDE4D3 protein.

## Materials and Methods

2,3,4,5,6-Pentafluorobenzoyl chloride, 4-(*N*,*N*-dimethylamino)pyridine (DMAP), *N*-hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 12-aminododecanoic acid, oleic anhydride, oleic acid, 1-oleoyl-*sn*-glycero-3-phosphate, 1-palmitoyl-*sn*-glycero-3-phosphocholine, glycerophosphorylcholine·$CdCl_2$, phospholipase $A_2$ ($PLA_2$) (EC 3.1.1.4) (*Naja mossambica* mossambica; porcine pancreas), phospholipase D (EC3.1.4.4) (*Streptomyces chromofuscus*, type 5; cabbage, type 1; peanut, type 2), and phosphatidic acid (PA) (from egg yolk lecithin) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). All the commercial enzyme preparations were used without further purification. [$^3$H]-1-oleoyl-2-lyso-*sn*-glycero-3-phosphatidic acid was obtained from NEN Life Science Products (Belgium).

Dry solvents were obtained as follows: chloroform by distillation over $P_2O_5$, methylene chloride, and dioxane by distillation over calcium hydride. They were then stored over 4 Å molecular sieves. Thin-layer chromatography (TLC) was performed using silica gel 60 F254 (Merck) plates. The solvents used for elution were (A) chloroform/methanol/water, 65:25:4 (vol/vol/vol) and (B) chloroform/methanol/acetic acid/water, 60:30:10:1 (vol/vol/vol/vol). To reveal the compounds, TLC plates were exposed to UV light, dipped in a solution of phosphomolibdic acid (1 g) in EtOH/$H_2SO_4$, and heated on a hot plate. Column chromatography was performed on silica gel (Kieselgel 60, 0.04−0.06 mm, Merck). Melting points were determined with a Kofler hot-stage melting-point apparatus. IR spectra were recorded on a Perkin-Elmer 1310 spectrometer. $^1$H NMR spectra were recorded with a Bruker AM200 spectrometer. Chemical shifts were given in ppm downfield from internal $Me_4Si$. Elemental analyses were performed by the "Service Central de Microanalyses du CNRS" (Solaize, France). Radioactive purity was checked after TLC with a Berthold model LB 511 analyzer. Reactions involving azides were carried out in subdued light by wrapping the flasks with aluminum foil.

**Synthetic Methods. Methyl Pentafluorobenzoate (2).** To 20 mL of methanol was added dropwise 6.0 g (26.1 mmol) of 2,3,4,5,6-pentafluorobenzoyl chloride with stirring. The mixture was refluxed for 1 h, the methanol was removed by evaporation, and the residue was dissolved in diethyl ether (20 mL). The solution was washed with saturated $NaHCO_3$ (2 × 15 mL), dried over $MgSO_4$, and evaporated to give 5.76 g (98%) of **2** as a colorless liquid. IR: 2958, 1742 (CO), 1645, 1331, 1240, 1010, 979 cm$^{-1}$.

Compound **3** was prepared according to the procedure described by Keana et al.[20]

**12-[*N*-(4-Azido−2,3,5,6-tetrafluorobenzoyl)]aminododecanoic Acid (4).** A total of 1.98 g (5.96 mmol) of succinimidyl 4-azido-2,3,5,6-tetrafluorobenzoate **(3)** and 1.41 g (6.56 mmol) of 12-aminoundecanoic acid was dissolved in dry dioxane (35 mL). The mixture was stirred at 50−60 °C for 4 h and concentrated. The resulting residue was diluted with ethyl acetate, and the resulting solution was washed with $H_2O$ (3 × 30 mL) and dried over anhydrous $MgSO_4$. After solvent evaporation the crude product was recrystallized from ethyl acetate to afford 1.44 g (90%) of **4**: mp 119−120 °C; IR($CHCl_3$) 3000, 2922, 2120 ($N_3$), 1678 (CO) cm$^{-1}$; $^1$H NMR ($CDCl_3$) $\delta$ 1.3 (M, 14H), 1.63 (m, 4H), 2.35 (t, $J$ = 7.5 Hz, 2H), 3.47 (q, 2H), 5.95 (se, NH). Anal. ($C_{19}H_{24}F_4N_4O_3$) N, C: calcd 52.78; found, 53.43. H: calcd 5.59; found 5.85.

**1-*O*-Palmitoyl-2-*O*-[12-[*N*-(4-azido-2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-*sn*-glycero-3-phosphocholine (6). Procedure A.** A mixture of fatty acid **4** (0.262 g, 0.606 mmol) and DCC (0.0747 g, 0.3636 mmol) was stirred in 2.5 mL of dry methylene chloride at room temperature overnight. The product mixture was filtered to remove urea. The solvent was evaporated to afford a crude product that was used directly in the synthesis of **6** without further purification. Examination of the filtrate by IR revealed the presence of fatty acid anhydride (1735 and 1805 cm$^{-1}$) and the absence of the parent carboxylic acid (CO, 1701 cm$^{-1}$).

A solution of the crude anhydride and DMAP (0.0042 g, 0.035 mmol) in dry chloroform (2 mL) was added under nitrogen to a suspension of 1-palmitoyl-lyso-PC **5** (14.4 mg, 0.029 mmol, dried twice just before use by evaporation of added benzene under reduced pressure). After being flushed with dry $N_2$, the sealed reaction mixture was stirred at room temperature in the dark for 40 h, the solvent was evaporated in vacuo, and the residue was purified by silica gel column chromatography, eluting first with chloroform/methanol (9:1, vol/vol) to remove fatty acid anhydride and DMAP and then with a chloroform/methanol gradient as described by Menger et al.[21] We thus obtained 12 mg (46%) of **6** as an amorphous solid. TLC $R_f$ = 0.28 (solvent A); $^1$H NMR ($CDCl_3$), $\delta$ 0.89 (t, $J$ = 6.5 Hz, 3H), 1.27 (M, 38H), 1.56 (m, 6H), 2.28 (q, $J$ = 7 Hz, 4H), 3.25 (s, 9H), 3.40 (q, $J$ = 7 Hz, 2H), 3.60−3.90 (m, 4H), 4.0−4.5 (m,4H), 5.15 (m, 1H), 6.80 (se, NH).

**Procedure B.** A mixture of 60.0 mg (0.121 mmol) of 1-palmitoyl-lyso-PC **5** (dried twice by evaporation of added benzene just before use), 209 mg (0.484 mmol) of **4**, 120 mg (0.484 mmol) of DCC, and 59.0 mg (0.484 mmol) of DMAP was suspended in 15 mL of dry dichloromethane and stirred under nitrogen in the dark for 40 h. The reaction mixture was diluted with 10 mL of methanol/water (19:1) and treated with 3 g of Dowex 50x8 ($H^+$ form) to remove the catalyst DMAP. The resin was removed by filtration and washed with methanol/chloroform/water (5:5:0.5 by volume) (2 × 10 mL). The solvent was evaporated under reduced pressure, and the crude product was subjected to silica gel column chromatography, eluting first with chloroform/methanol (9:1 vol/vol) to remove fatty acid and other nonpolar compounds and then with a chloroform/methanol gradient (50 mL of $CHCl_3$, 90 mL of $CHCl_3$/10 mL of $CH_3OH$, 80 mL of $CHCl_3$/20 mL of $CH_3OH$, 50 mL of $CHCl_3$/50 mL of $CH_3OH$, 20 mL of $CHCl_3$/80 mL of $CH_3OH$) to give phospholipids. Fractions containing **6** were pooled, and the solvents were evaporated. The residual water was removed by azeotropic evaporation in a rotary evaporator with ethanol/benzene (4:1 vol/vol). We thus obtained 93.7 mg (85.2%) of **6** as an amorphous solid.

**1-*O*-Palmitoyl-2-*O*-[12-[*N*-(4-azido−2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-*sn*-glycero-3-phosphate (7).** The phosphatidic acid precursor **6** (7mg) was sonicated in 2 mL of 40 mM sodium acetate, pH 5.6, containing 80 mM $CaCl_2$. Diethyl ether (2 mL) and 125 U of phospholipase D (from S. chromofuscus) were added, and the reaction mixture, protected from light, was stirred vigorously at 30−35 °C for 5 h. Monitoring of the reaction by TLC indicated after 5 h the disappearance of phosphatidylcholine and the occurrence of a less polar compound. The ether was removed under reduced pressure, and the residue was extracted with 3 × 10 mL chloroform/methanol (2:1). Combined chloroform extracts were washed with water (3 × 20 mL) and dried over $Na_2SO_4$. Removal of the solvent gave the crude product, which was further purified by preparative TLC: $R_f$ = 0.8 (solvent B), in comparison with PC **6** ($R_f$ = 0.15) (3.2 mg; 50% yield). $^1$H NMR ($CDCl_3$) $\delta$ 0.89 (t, $J$ = 6.5 Hz, 3H), 1.27 (M, 38H), 1.56 (M, 6H), 2.28 (q, $J$ = 7 Hz, 4H), 3.45 (q, $J$ = 7 Hz, 2H), 3.70−3.90 (m, 2H), 4.15 (m, 1H), 4.45 (m, 1H), 5.25 (m, 1H).

**1,2-*O*-Di[12-[*N*-(4-azido-2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-*sn*-glycero-3-phosphocholine (9).** Commercial *sn*-glycero-3-phosphorylcholine·$CdCl_2$ (GPC−$CdCl_2$, **8**) (100 mg, 0.227 mmol) was made anhydrous by repeated evaporation of added dry benzene. The dry GPC−$CdCl_2$ complex was then suspended in 3.0 mL of anhydrous dichloromethane, to which 392 mg (0.908 mmol) of fatty acid **4**, 187 mg (0.908 mmol) of DCC, and 111 mg (0.908 mmol) of DMAP were added. The mixture was stirred under nitrogen at room temperature (about 25 °C) in the dark for 40 h. The solvent was removed under reduced pressure. Purification by silica gel column chromatography (first, chloroform/methanol, 9:1; second, the chloroform/methanol gradient as above) afforded the desired product **9** as an amorphous solid (207 mg, 74%). TLC $R_f$ = 0.4 (solvent A), $^1$H NMR ($CDCl_3$) $\delta$ 1.28 (M, 28H),

1.59 (m, 8H), 2.25 (m, 4H), 3.25 (s, 9H), 3.30 (m, 4H), 3.55 (m, 2H), 3.90 (m, 2H), 4.10−4.50 (m, 3H), 5.20 (m, 1H). HRMS (FAB) calcd for $C_{46}H_{64}F_8N_9O_{10}P$ ($M + H^+$) 1086.4464, found 1086.4462.

**1-*O*-[12-[*N*-(4-Azido-2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-2-lyso-*sn*-glycero-3-phosphocholine (10).** To a solution of 110 mg (0.087 mmol) of PC **9** in 1 mL of methanol was added 1.5 mL of 100 mM sodium borate buffer, with 100 mM $CaCl_2$ (pH 8.0) and 500 units of phospholipase $A_2$ (*Naja mossambica* mossambica). The mixture was stirred vigorously at 38 °C. The reaction was monitored by TLC, and after 5 h it was judged to be complete by the presence of lyso-PC **10** and free fatty acid and the complete disappearance of PC **9**. To remove the fatty acid, the reaction mixture was extracted with ether (3 × 10 mL). The aqueous phase was then extracted with chloroform/methanol (2:1) (3 × 20 mL). The chloroform/methanol extract was dried over $Na_2SO_4$, and the solvents were evaporated. The residue was subjected to silica gel column chromatography, eluting with chloroform/methanol/water (65:25:4). Fractions containing lyso-PC **10** were collected, and the solvents were removed under reduced pressure. The residual water was removed by azeotropic evaporation in a rotary evaporator with ethanol/benzene (4:1 vol/vol). We thus obtained 54 mg (73%) of **10**. TLC $R_f$ = 0.2 (solvent A), in comparison with PC **9** ($R_f$ = 0.4). $^1$H NMR ($CD_3OD$) $\delta$ 1.30 (M, 14H), 1.60 (m, 4H), 2.35 (t, $J$ = 7.5 Hz, 2H), 3.20 (s, 9H), 3.35 (m, 2H), 3.60 (m, 3H), 3.80−4.10 (m, 4H), 4.10−4.40 (m, 2H).

**1-*O*-[12-[*N*-(4-Azido-2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-2-*O*-oleoyl-*sn*-glycero-3-phosphocholine (11).** Lyso-PC **10** (47 mg, 0.055 mmol) was made anhydrous by evaporation of added benzene and was then dissolved in 3.0 mL of dry chloroform. To this solution, 300.8 mg (0.55 mmol) of oleic anhydride and 26.84 mg (0.22 mmol) of DMAP were added. The mixture was stirred at room temperature in the dark for 5 h. Removal of the solvent under reduced pressure left a residue that was purified by silica gel chromatography. We first eluted with chloroform/methanol (9:1) to remove unreacted fatty acid anhydride and DMAP and then with chloroform/methanol/water (65:25:4). Fractions containing the PC **11** were pooled. Evaporation of the solvent left a light-yellow residue, which was dried twice by evaporation of added benzene (3 mL). We thus obtained 58 mg (94%) of **11**. TLC $R_f$ = 0.43 (solvent A), in comparison with lyso-PC **10** ($R_f$ = 0.14). $^1$H NMR ($CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H), 1.32 (M, 34H), 1.59 (m, 6H), 2.0 (m, 4H), 2.30 (m, 4H), 3.30 (s, 9H), 3.40 (m, 2H), 3.70 (m, 2H), 3.90 (m, 2H), 4.15 (d × d, $J$ = 12, $J$ = 7.5 Hz, 1H), 4.20−4.50 (m, 3H), 5.15 (m, 1H,), 5.30 (m, 2H), 6.70 (t, $J$ = 4 Hz, NH). Anal. ($C_{45}H_{74}F_4N_5O_9P$) C: calcd 57.74; found, 58.05. H: calcd 7.97; found, 8.04. N: calcd 7.48; found, 7.60.

**1-*O*-[12-[*N*-(4-Azido−2,3,5,6-tetrafluorobenzoyl)]aminododecanoyl]-2-*O*-oleoyl-*sn*-glycero-3-phosphate (12).** The procedure used for the preparation of PA **12** was similar to that described above for the preparation of PA **7** except that PC **11** was used instead of PC **6** (yield, 61%). TLC $R_f$ = 0.8 (solvent B), in comparison with PC **11** ($R_f$ = 0.15); $^1$H NMR ($CDCl_3$) $\delta$ 0.90 (t, $J$ = 6.5 Hz, 3H), 1.25 (M, 34H), 1.55 (m, 6H), 2.0 (m, 4H,), 2.30 (m, 4H), 3.45 (m, 2H), 3.70 (m, 2H), 4.10−4.40 (m, 2H), 5.15 (m, 1H), 5.30 (m, 2H). Anal. ($C_{40}H_{62}F_4N_4O_9P$) C, H, N.

**1-*O*-Oleoyl-2-*O*-[12-[*N*-(azido-2,3,5,6-tetrafluorobenzoyl)]-aminododecanoyl]-*sn*-glycero-3-phosphocholine (14).** The procedure used for the preparation of PC **14** was similar to that described for PC **9** starting from 1-oleoyl-lyso-PC **13** (yield, 60%). TLC $R_f$ = 0.4 (solvent A); $^1$H NMR ($CDCl_3$ + $CD_3OD$) $\delta$ 0.85 (t, $J$ = 7.5 Hz, 3H), 1.30 (M, 34H), 1.0 (m, 6H), 2.0 (m, 4H), 2.30 (m, 4H), 3,25 (s, 9H), 3.40 (m, 2H), 3.65 (m, 3H), 3.95 (m, 2H), 4.10 (d × d, $J$ = 12, 7 Hz, 1H), 4.25−4.0 (m, 2H), 5.20 (m, 1H), 5.35 (m,2H). Anal. ($C_{45}H_{74}F_4N_5O_9P$) C, N, H: calcd 7.97; found, 8.06.

**1-*O*-Oleoyl-2-*O*-[12-[*N*-(azido-2,3,5,6-tetrafluorobenzoyl)]-aminododecanoyl]-*sn*-glycero-3-phosphate (15).** We used the same procedure as that described to obtain product **7** (yield, 62%). TLC $R_f$ = 0.8 (solvent B); $^1$H NMR ($CD_3OD$) $\delta$ 0.90 (t, $J$ = 6.5 Hz, 3H), 1.30 (M, 34H), 1.55 (m, 6H), 2.0 (m, 4H), 2.30 (m, 4H), 3.35 (m, 2H), 3.70−3.90 (m, 2H), 4.10−4.40 (m, 2H), 5.25 (m, 1H), 5.35 (q, 2H). Anal. ($C_{40}H_{62}F_4N_4O_9P$) C, H: calcd 7.30; found, 7.42. N: calcd 6.59; found, 6.65.

**Synthesis of 2-Azido-[$^3$H]phosphatidic Acid (16).** Tritium was introduced by acylating [$^3$H]-1-oleoyl-2-lyso-*sn*-glycero-3-phosphatidic acid (50 $\mu$Ci, 86 nmol) with acid **4** (8.6 $\mu$mol), DMAP (8.6 $\mu$mol), and DCC (8.6 $\mu$mol) in 2 mL of anhydrous chloroform under the conditions described previously (Scheme 5). The radiolabeled PA **16** was purified by TLC (solvent A). After extraction with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (5/5/0.1/1), the purity was checked by radioscanning a TLC plate with a Berthold counter. The yield was 50%, and the specific activity was about 0.6 Ci/mmol.

**Preparation of Recombinant PDE4D3.** MA-10 Leydig tumor cells were cultivated in Waymouth MB752/1 medium modified as previously reported.[22] Rat PDE4D3 was expressed in MA-10 cells by calcium phosphate transfection with 10−15 $\mu$g of pCMV5 containing the corresponding cDNA.[23] Mock-transfected cells were treated in the same way but did not receive plasmidic DNA. At 24 h after transfection, the cells were harvested in 40 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM EGTA, 50 mM benzamidine, 0.5 $\mu$g/mL leupeptin, 0.7 $\mu$g/mL pepstatin, 4 $\mu$g/mL aprotinin, 10 mg/mL soybean trypsin inhibitor, and 2 mM phenylmethylsulfonyl fluoride. Cells were homogenized and centrifuged for 10 min at 14 000*g* at 4 °C. Soluble extracts were diluted to 33% with ethylene glycol and stored at −20 °C.

Untransfected or mock-transfected MA10 cells express no immunodetectable amounts of type 4PDE, and their basal activity was not sensitive to PA stimulation.[10] Transfection with pCMV5−PDE4D3 plasmid routinely increased PDE specific activity by 20- to 100-fold above the basal level. Thus, the endogenous PDE activity accounted for <5% of the recombinant PDE activity.

**Phosphodiesterase Assay.** PDE activity was measured using 1 $\mu$M cAMP as the substrate, according to the method of Thompson and Appleman.[24] Samples were assayed in a total volume of 400 $\mu$L of reaction mixture including 1 mM $MgCl_2$, 0.5 mg/mL gelatin, 40 mM Tris-HCl, pH 8.0, 125 mM 2-mercaptoethanol, and 0.1 $\mu$Ci [$^3$H]cAMP. All assays were done in triplicate.

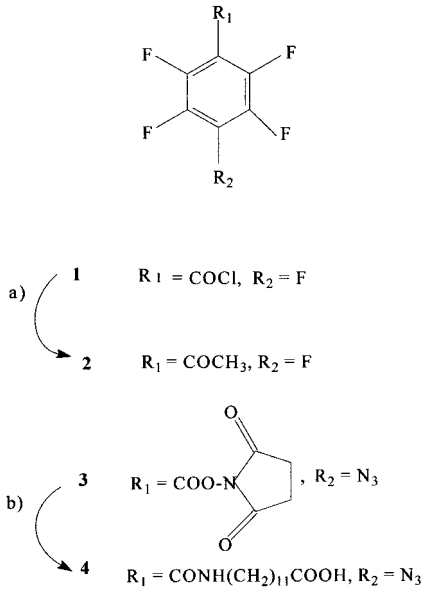
**Preparation of Phospholipid Suspensions.** Phospholipid stock solutions in chloroform were evaporated under a nitrogen flux. Phospholipids were then resuspended in 40 mM Tris-HCl, pH 8.0, and suspensions were obtained by sonicating the lipids with a probe sonicator (three cycles of 10 s). Lipid suspensions were then appropriately diluted in the PDE assay buffer.

**Photolabeling of PDE4D3.** A total of 30 $\mu$g of proteins of recombinant PDE4D3 prepared in MA10 cells was incubated in the presence or absence of 2-azido[$^3$H]PA **16** in a total volume of 60 $\mu$L for 15 min on ice in the dark. They were then irradiated at a distance of 8 cm with an ultraviolet lamp (Bioblock 254/365) at 254 nm wavelength for 15 min. Samples were then diluted in Laëmmli buffer and submitted to SDS−PAGE on a 10% acrylamide gel; the proteins were transferred onto an Immobilon P membrane (Millipore). PDE4D3 was immunodetected with a PDE4-specific polyclonal antibody (K116, 1:500) as described in ref 25. A second antibody incubation was carried out with antirabbit IgG conjugated to peroxidase, and immunoreactive bands were detected by the ECL method (Amersham Pharmacia Biotech). The membrane was cut into 5 mm wide strips, which were incubated in scintillation vials with 500 $\mu$L of Soluene (Packard) for 30 min before 5 mL of scintillation solution (IonicFluor) was added, and counted.

## Results and Discussion

**Design and Preparation of Photoreactive Phosphatidic Acid Analogues.** Numerous photoactivatable phospholipids have been prepared and applied as general labeling reagents or as photoaffinity probes. Most

**Scheme 1**[a]




[a] (a) MeOH (98%). (b) $NH_2(CH_2)_{11}COOH$ (90%).

of them are PCs carrying the photoactivatable group linked to the *sn*-2 fatty acyl chain or to the polar headgroup.[26] Since a study of the structural requirements of phospholipid−PDE interaction previously performed in our laboratory had shown that the nature and position of fatty acyl moieties in the PA molecule markedly influence their ability to activate the PDE enzyme,[27] we have synthesized PA analogues bearing the photoactivatable group either at the sn-1 or at the sn-2 position. Following some pioneering work,[20,28,29] polyfluorinated aromatic azides have been widely used for photoaffinity labeling. These perfluorinated arylazides would generate a more reactive singlet nitrene and offer improved cross-linking efficiency relative to their simple phenylazide counterpart. Consequently in the present work we selected a 4-azidotetrafluorobenzylacyl group as a photoreactive moiety that was expected to link covalently to the neighboring amino acid residues on the PDE peptide chain. We synthesized phosphatidic acid analogues 1-azidoPA (**12**) and 2-azidoPA (**7** and **15**), which bear this azido group at the *sn*-1 and *sn*-2 fatty acyl chains, respectively.

The synthetic routes used for the preparation of 1-azidoPA and 2-azidoPA are outlined in Schemes 1−5. *N*-Succinimidyl 4-azidotetrafluorobenzoate (**3**) was prepared as described by Keana et al.[20] and reacted with 12-aminododecanoic acid to afford 12-[*N*-(4-azido-2,3,5,6-tetrafluorobenzoyl)]aminododecanoic acid (**4**). Acylation of 1-palmitoyl-lyso-PC or 1-oleoyl-lyso-PC with azido fatty acid **4** using DCC as the condensing agent and DMAP as a base produced the corresponding phosphatidylcholine 2-azidoPC **6** or **14**, which were further hydrolyzed with PLD (from peanut or S.chromofuscus) to give phosphatidic acid analogue 2-azidoPA (**7** or **15**).

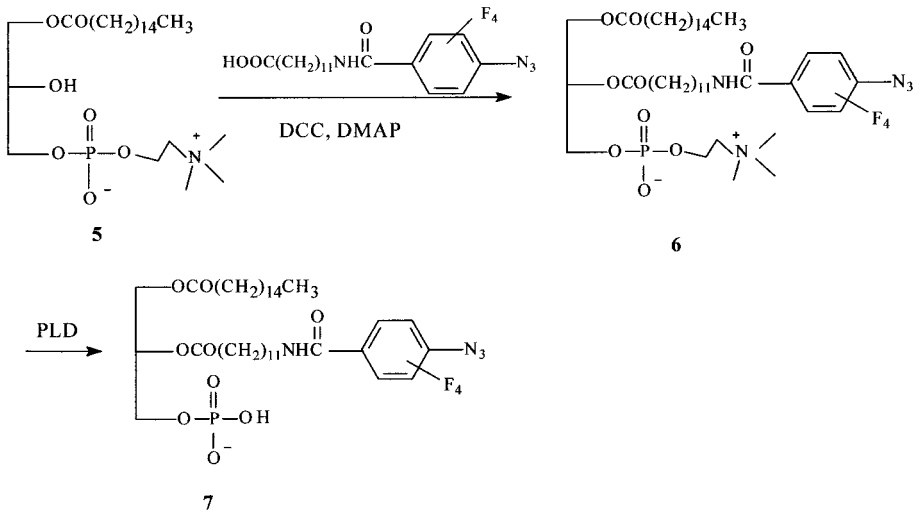
We first attempted to synthesize a 1-azidoPA analogue by treatment of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine with lipase (EC 3.1.1.3) from Rhizopus arrhizus, which exhibits $PLA_1$ activity, to obtain the corresponding 2-arachidonoyl-lysophospholipid. A subsequent esterification with azido fatty acid **4** would produce 1-azidoPC. However, we observed a rapid isomerization of 2-arachidonoyl-lysophospholipid into 1-arachidonyl-2-lyso-PC because of a weak stability of 2-lyso-PC at the pH of the following esterification.[30] So we used another method to synthesize the 1-azido PA analogue. The cadmium chloride complex of glycerophosphocholine (GPC·$CdCl_2$) was first diacylated by azido fatty acid **4**, leading to 1,2-diazidoPC **9**, which was then converted into 2-lysophospholipid **10** by treatment with phospholipase $A_2$ (from *Naja mossambica* mossambica). A subsequent acylation with oleic anhydride yielded 1-azidoPC **11**, which was then hydrolyzed with PLD (from *S. chromofuscus*) to give the phosphatidic acid analogue 1-azidoPA **12**.

**Effects of Reaction Conditions on Acylation.** After the pioneering work of Höfle et al.,[31] Gupta et al. introduced DMAP as a catalyst for acylation of lysolecithins or *sn*-glycero-3-phosphorylcholine (GPC) with fatty acid anhydrides.[32] This mild and efficient procedure for the partial synthesis of symmetrical 1,2-diacyl or mixed diacylglycerophospholipids has been predominantly used in lipids synthetic chemistry. In the present work, the acylation reaction of lyso-PC **10** using a 10-fold molar excess of oleic anhydride in the presence of DMAP[33] could be achieved with 94% yield at room temperature for 3 h. In contrast, we obtained a relatively modest yield (46%) for the acylation of 1-palmitoyl-lyso-PC **5** to obtain **6**, by using 10 mol equiv of anhydride (prepared by reaction of 20 mol equiv of fatty acid **4** and 11 mol equiv of DCC in dry methylene chloride prior to use) and 1.2 mol equiv of DMAP in dry chloroform at room temperature for 40 h. Fortunately when the acylation was conducted with 1 mol equiv of GPC·$CdCl_2$ or lyso-PC, 4 mol equiv of fatty acid **4**, 4 mol equiv of DCC, and 4 mol equiv of DMAP as described in Material and Methods, **9** and **14** were obtained in 74% and 60% yield, respectively.

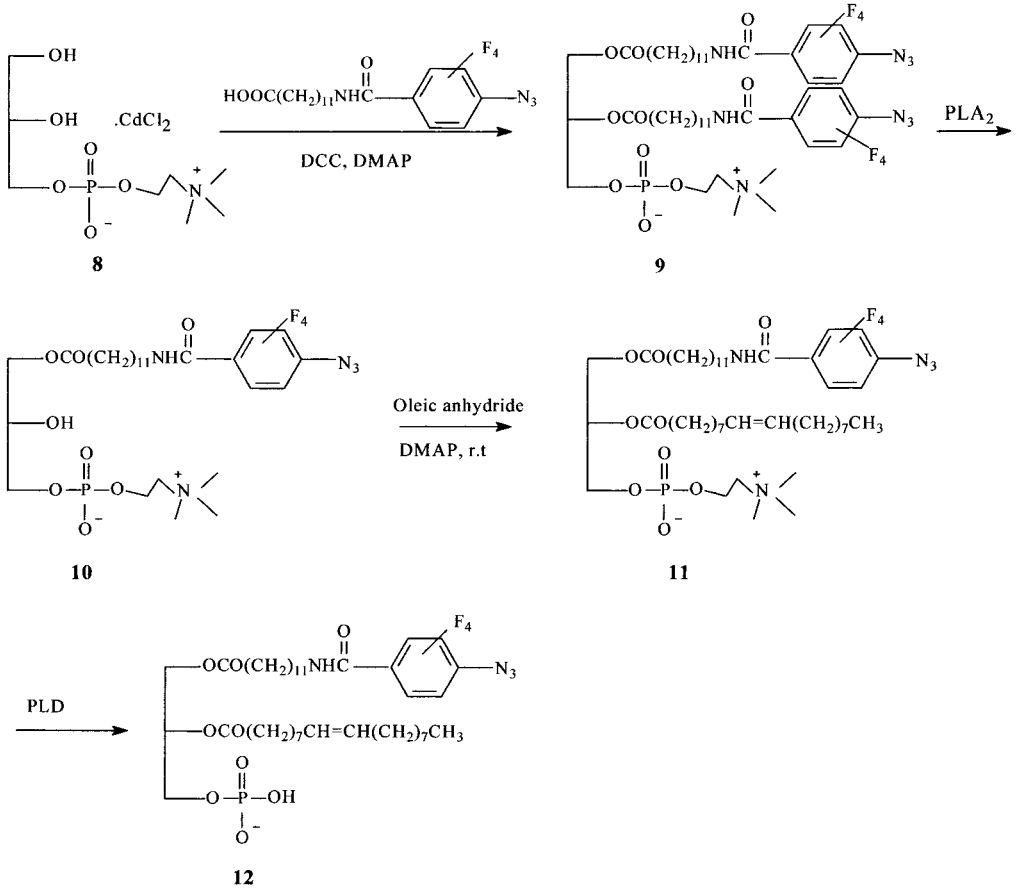
**Activity of Phospholipase $A_2$ and Phospholipase D on 1,2-DiazidoPC.** Phospholipase $A_2$ catalyzes the hydrolysis of fatty acid ester bonds at the *sn*-2 position of naturally occurring 1,2-diacyl-*sn*-glycerophospholipids, in a stereo- and regiospecific manner. In addition to the polar headgroup present at the *sn*-3 position of the glycerophospholipid substrate, the pattern of acyl chains at the *sn*-1 and *sn*-2 positions has a crucial influence on the enzymatic activity. During the preparation of lyso-PC **10**, we have found that $PLA_2$ from porcine pancreas was unable to catalyze the hydrolysis of 1,2-diazidoPC. No significant hydrolysis was observed even when we changed the reaction conditions by raising the temperature to 38 °C or by adding sodium deoxycholate or ether to the reaction mixture. However, the enzyme preparation from *Naja mossambica* mossambica could convert 1,2-diazidoPC into the corresponding lyso-PC **10** at 38 °C for 5 h. It is worth noting that the hydrolysis reaction must be conducted at 38 °C or near this temperature, the rate of hydrolysis being negligible at room temperature. We also found that phospholipase D from peanut, *S. chromofuscus*, or cabbage sources could not catalyze the conversion of 1,2-diazidoPC **9** to the corresponding PA, although they efficiently hydrolyzed 1-azidoPC **11** and 2-azidoPCs **6** and **14**.

**Activation of PDE4D3 by Phosphatidic Acid Analogues.** We have previously shown that the "long"

**Scheme 2**

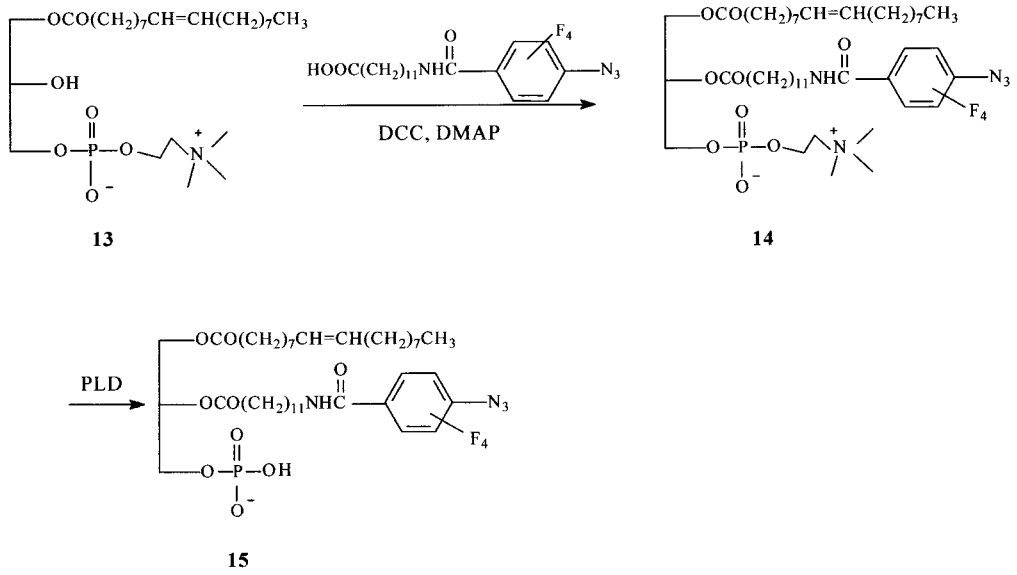



**Scheme 3**

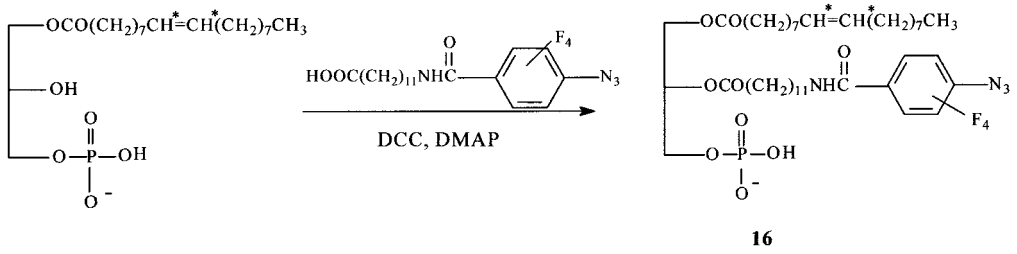



isoforms of PDE4 carrying an N-terminal regulatory region, particularly the isoform PDE4D3, can be specifically activated by acidic phospholipids, PA being the most potent activator.[10] The influence of PA and the corresponding PC analogues on the activity of recombinant cAMP-specific PDE4D3 was determined by using 100 $\mu$g/mL of the different phospholipids. PA from egg yolk was used as a reference compound because it was previously shown to be a selective activator of PDE4D3. As shown in Figure 1, all three phosphatidylcholines were totally inactive, whereas the three photoactivatable PA analogues stimulated enzymatic activity to various extents. The examination of dose−response curves of PDE activation (Figure 2) showed that PA **12** and egg yolk PA induced similar maximal activation, while PA **15** and PA **7** were less efficient. The $EC_{50}$ values of the three PA analogues were comparable (similar to 20 $\mu$g/mL, approximatively $2 \times 10^{-5}$ M), i.e., slightly higher than the $EC_{50}$ of PA of natural origin (10 $\mu$g/mL). Thus, the introduction of a bulky photoactivatable group on one of the acyl chain of PA did not markedly affect the affinity of the phospholipid for PDE4D3. However, the position of the modified fatty acid influenced the efficacy of the compound for activat-

**Scheme 4**




**Scheme 5**



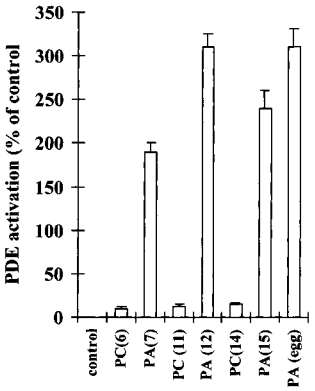




**Figure 1.** Effect of various phospholipids on PDE4D3 activity. The activity of recombinant PDE4D3 was assayed in the absence (control) or presence of 100 $\mu$g/mL concentrations of various phospholipids. Results are expressed as a percentage of the control value. Results are means ± SD of three independent experiments.

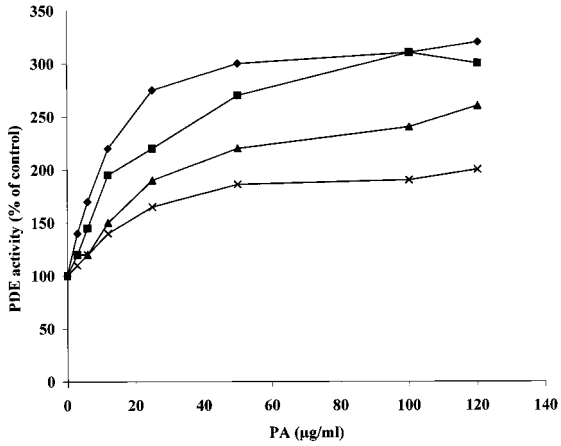



**Figure 2.** Effect of the different PA on PDE4D3 activity. The activity of recombinant PDE4D3 was assayed in the absence (control) or presence of increasing concentrations of various PA. Results are expressed as a percentage of the control value without any addition (100%): ◆, egg yolk PA; ■, PA **12**; ▲, PA **15**; ×, PA **7**.

ing the enzyme. In agreement with our previous observation that the presence of an unsaturated fatty acid at the *sn*-2 position is a prerequisite for PA to activate phosphodiesterase,[27] PA **12** (2-oleoyl) was the most efficient activator among the three analogues. In this respect, it is rather surprising that compounds **7** and **15**, which carry 4-azidotetrafluorobenzoic acyl substituted chains at the *sn*-2 position, displayed activating properties. This can probably be explained by the fact that the bulky 4-azidotetrafluorobenzoic acyl group plays a double-bond-like function in disturbing the regular arrangement of phospholipid molecules in vesicles, and subsequently it influences the phase-transition temperature.

**Specific Covalent Labeling of PDE4D3 by 2-AzidoPA.** Since the PA analogues 1-azido-PA and 2-azido-PA have a certain affinity for the enzyme, they can be suitable candidates as photoaffinity labeling agents. We have synthesized compound **16**, a radiolabeled form of the photoreactive compound **15**. We choose to synthesize an analogue of **15** rather than **12**, which displayed

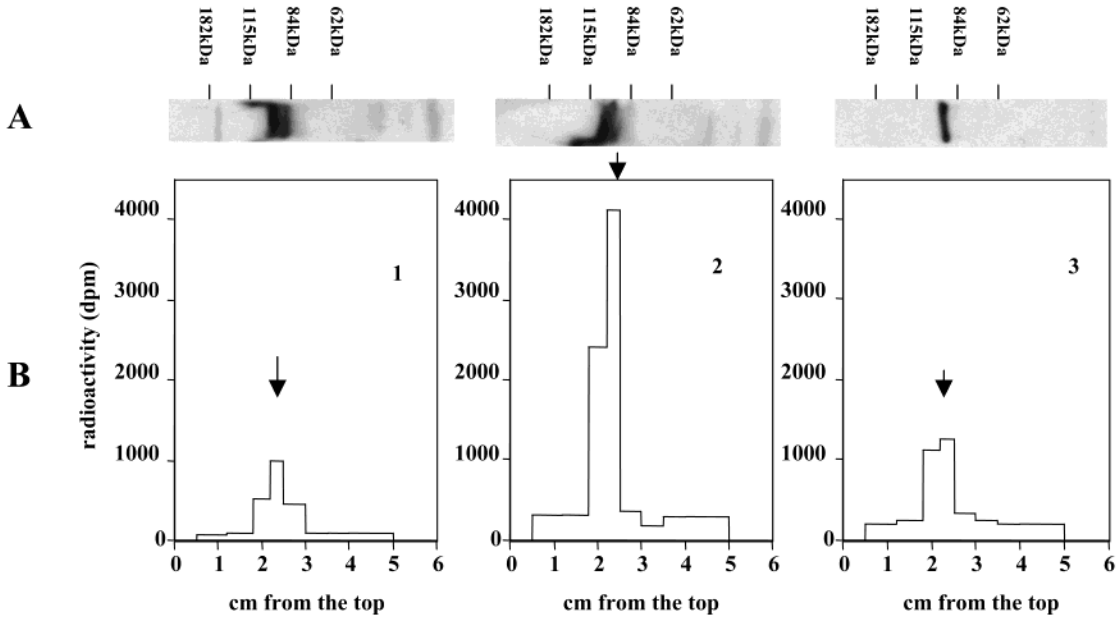



**Figure 3.** Photoaffinity labeling of PDE4D3 with 2-azido-[$^3$H]PA. (A) Position of the PDE4D3 band is determined by Western blotting analysis using K116 antibody: (lane 1) PDE4D3 incubated 15 min with 50 kBq 2-azido[$^3$H]PA without irradiation; (lane 2) PDE4D3 incubated 15 min with 50 kBq 2-azido-[$^3$H]PA with 15 min UV irradiation; (lane 3) PDE4D3 incubated with 50 kBq 2-azido-[$^3$H]PA and 100 $\mu$M PA from egg yolk, with 15 min irradiation. (B) Radioactivity profile corresponds to each lane on the membrane. Membrane was cut into 5 mm slices, and radioactivity was determined by scintillation counting.

higher PDE-activating effects, because of the possibility of obtaining a radiolabeled analogue in markedly higher yield by directly acylating [$^3$H]-1-oleoyl-2-lyso-*sn*-glycero-3-phosphatidic acid.

Irradiation experiments aimed at evaluating the ability to specifically label the PDE4D3 protein were then performed. The crude recombinant phosphodiesterase preparation was incubated with [$^3$H]PA **16**, in the presence or absence of an excess of unlabeled PA (from egg yolk), and submitted or not to UV irradiation. Proteins were then fractionated by SDS−polyacrylamide gel electrophoresis. Western blotting using an antibody recognizing specifically the PDE4 isoforms and radioactivity counting of the blotting membrane showed that UV irradiation induced the comigration of radioactive label with the protein band corresponding to PDE4D3 (93 ± 1.1 kDa) (Figure 3). In the absence of irradiation, the amount of radioactivity linked to PDE4D3 was markedly lower, showing the photodependence of the labeling (Figure 3, lane 1). Furthermore, the addition of an excess of unlabeled PA strongly decreased the radioactive labeling of PDE4D3 (Figure 3, lane 3), showing that binding of compound **16** to PDE4D3 was displaced by PA from egg yolk and demonstrating that both compounds competed for the same site(s) on the enzyme.

On the whole, we describe in this study the synthesis of new analogues of the natural phospholipid phosphatidic acid carrying the photoactivatable 4-azidotetrafluorobenzylacyl group. We obtained three analogues that retain, to various extents, the ability of PA to activate the cAMP-hydrolyzing enzyme PDE4D3. Compound **16**, which is substituted at the *sn*-1 position by oleic acid and at the *sn*-2 position by azidotetrafluorobenzoylaminododecanoic acid, was synthesized in a radioactive form. It was shown to specifically label the PDE4D3 protein in a crude recombinant enzyme preparation. After irradiation, the radiolabel was found at the level of the enzyme band following an electrophoresis in denaturing conditions, which shows that the compound was covalently linked to the protein. Labeling of PDE4D3 involved photoactivation of the azido group carried by the PA analogue because it was markedly enhanced by UV irradiation. These results confirm the presence of specific PA binding site(s) on the PDE4D3 protein and open the possibility of using this labeling technique for the characterization of these sites. In particular, covalent labeling of tryptic fragments of the enzyme should allow for the determination of the amino acid residues, which are involved in PA−PDE4 interaction. It could help to determine the precise sequence of the PA binding site previously delimited by deletion mutation studies.[13] More generally, photoactivatable PA analogues appear as promising tools for the study of the PA binding site carried by several proteins playing major roles in cell signaling, such as raf-1 kinase[16] and phospholipase C $\delta$3.[18] Furthermore, the demonstration that PA analogues carrying bulky substituents retain selective binding to PDE4 and have the ability to activate these enzymes suggests their possible use as irreversible or reversible PDE4 activators of pharmacological interest. This is in contrast to PDE4 inhibitors, which have been the subject of intensive studies leading to the characterization of compounds highly promising for the treatment of various disorders such as allergy, inflammation, etc.[7−9,34] Up to now, drugs that activate these enzymes and thus influence cell responses by decreasing intracellular cAMP levels have not been taken into consideration.

PA analogues might thus constitute the prototypes of a new category of drugs, the pharmacological activity of which deserves evaluation.

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