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Syntheses of D-*myo*-inositol 1,4,5-trisphosphate affinity ligands *

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ABSTRACT

A mixture of 2,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol and 1,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol, obtained during our synthesis of D-*myo*-inositol 1,4,5-trisphosphate [C.E. Ballou and W. Tegge, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 94–98], was separated after tetrahydropyranylation of the free hydroxyl group in each. 2,3,6-Tri-*O*-benzoyl-4,5-di-*O*-benzyl-1-*O*-(tetrahydro-2-pyranyl)-D-*myo*-inositol was debenzylated and the two free hydroxyl groups were phosphorylated by a dibenzyl phosphoramidite procedure. The tetrahydropyranyl group was then removed, and phosphorylation at position 1 with benzyl 3-(benzyloxycarbonylamino)propyl di-*N*-isopropylphosphoramidite, followed by oxidation and deprotection, provided 1-[3-aminopropoxy(hydroxy)phosphinyl]-D-*myo*-inositol 4,5-bisphosphate. This compound was coupled to activated agarose to prepare an affinity matrix for the isolation of D-*myo*-inositol 1,4,5-trisphosphate-binding proteins, and it was coupled to 4-azido-2-hydroxybenzoic acid to give a product that was labeled with ^{125}I to prepare a photoactivable derivatizing reagent. The new derivatives retain significant biological activity as assessed by their ability to stimulate the release of stored Ca^{2+} from the endoplasmic reticulum of permeabilized rat basophilic leukemia cells.

INTRODUCTION

The covalent attachment of ligands to insoluble matrices or to derivatizing agents has played an important role in the isolation and characterization of specific receptors. Recent interest in the D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) binding proteins has stimulated research leading to the preparation of affinity ligands to this second messenger. Hirata and coworkers have coupled $InsP_3$ with a derivatizing group by the action of a carbodiimide reagent[1–4], while we[5] have

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described syntheses of model affinity ligands based on the cyclohexanediol bisphosphate group as an analog of $InsP_3$.

We have now applied a similar strategy to the synthesis of a derivative of $InsP_3$ by utilizing chiral intermediates available from our earlier synthesis of this isomer[6]. The key target in this synthesis is 1-[3-aminopropoxy(hydroxy)phosphinyl]-D-*myo*-inositol 4,5-bisphosphate, which was prepared by a 6-step procedure. This compound has been coupled to agarose beads to provide an affinity matrix and to the photoactivable reagent 4-azido-2-hydroxybenzoic acid, which was then labeled with ^{125}I. These derivatives retain significant calcium ion-releasing activity, which suggests they should be effective affinity ligands for the isolation of $InsP_3$-binding proteins, and their utility is described elsewhere[7].

EXPERIMENTAL

Materials.—The mixture of tribenzoyldibenzyl-*myo*-inositols **1a** and **1b** was obtained in a ratio of 3:1 from D-pinitol as described previously[6]. When the numbering of the parent inositol[8] is such as to lead to the designation L-*myo*-inositol, **1a** is 1,2,4-tri-*O*-benzoyl-5,6-di-*O*-benzyl-L-*myo*-inositol, and **1b** is 1,3,4-tri-*O*-benzoyl-5,6-di-*O*-benzyl-L-*myo*-inositol. Named as derivatives of D-*myo*-inositol, **1a** is 2,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol, and **1b** is 1,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol. To emphasize their relation to D-*myo*-inositol 1,4,5-trisphosphate, we have chosen to name the intermediates as derivatives of D-*myo*-inositol, as recommended by the Nomenclature Committee of the International Union of Biochemistry[9].

m-Chloroperoxybenzoic acid, used to oxidize phosphite ester intermediates, was from CTC Organics, Atlanta. Reacti-Gel (1,1′-carbonyldiimidazole-activated, 6% crosslinked beaded agarose) and succinimido 4-azido-2-hydroxybenzoate were from Pierce, Rockford, IL. Most other organic reagents were from the Aldrich Chemical Co. Radioactive, carrier-free $Na^{125}I$ (1 mCi, pH 7–11) was from Amersham. Ethyl acetate was made acid-free by extracting it 3 times with 10% K_2CO_3 solution and once with water.

Methods.—All reactions involving uncharged inositol compounds were monitored by analytical high-performance liquid chromatography (HPLC) on a Supelco LC18 (5 μm) stainless steel column (0.4 × 25 cm) by elution with MeOH–water at a flow rate of 1.2 mL/min. An LKB 2138 Uvicord S, equipped with a microflow cell and a 254-nm filter was used to monitor the effluent. For preparative HPLC, a stainless steel column (5 × 50 cm) (HT Chemicals, St. Louis, MO) was packed manually with Merck LiChroprep RP-18 (25–40 μm) (EM Scientific, Cherry Hill, NJ) and eluted with MeOH–water at flow rate of 25 mL/min. The UV detector was equipped with a preparative flow cell, and solvents were mixed and delivered by a Waters Prep LC3000 system. Ion exchange was performed on a diethylaminoethyl adsorbent (DEAE Sephadex A-25 from the Sigma Chemical Co.).

Melting points were determined in capillaries on a Thomas-Hoover apparatus and are uncorrected. Optical activity was measured on a Bendix 1100 Automatic Polarimeter with a Hg lamp (546 nm). ^{31}P-NMR spectra, referenced to external 85% phosphoric acid, were determined on a Bruker 400 MHz spectrometer. ^{1}H-NMR spectra, referenced to internal Me_4Si (0.0 ppm) or acetone (2.225 ppm), were determined on a Bruker 500 MHz spectrometer in the Chemistry Department on this campus. Combustion analyses were performed in the Chemistry Department Microchemical Analysis Laboratory. Where elemental analyses are not provided, purity was assessed from the ^{31}P-NMR spectra, copies of which were submitted to the editor for evaluation.

Hydrogenations were carried out with H_2 gas at 1 atm in a boat mounted on an Eberbach reciprocal shaker. For acid-sensitive compounds that could not be handled in acetic acid, palladium hydroxide-on-carbon (Pearlman's catalyst, Aldrich) was used as the hydrogenation catalyst. All work with the light-sensitive azidosalicylic acid compounds was done under dim red light in a photographic laboratory. Phosphate was determined according to Ames[10]. Biological Ca^{2+}-release activity was determined in a system employing saponin-permeabilized rat basophilic leukemia cells as described earlier[11,12].

3-(Benzyloxycarbonylamino)-1-propanol.—To a vigorously stirred solution of 3-amino-1-propanol (7.5 g, 100 mmol) in 75 mL of 2 M NaOH was added 80 mL of 35% benzyl chloroformate in toluene at −10°. After the solution was stirred for 2 days at 25° a ninhydrin test for amine was negative, and the mixture was neutralized with M HCl. The solvents were evaporated, the residue was taken up in MeOH, the solution was filtered to remove precipitated NaCl, and then fractionated by preparative HPLC with 40:60 MeOH–water as eluent. The product was eluted after 50 min to yield 16.4 g (78%) of a white solid, ^{1}H-NMR ($CDCl_3$): δ 7.28–7.39 (5 H, m, Ar), 5.05–5.18 (3 H, br. s overlayed with sharp s, N*H* and PhCH_2), 3.63–3.69 (2 H, t, *J* 5.6 Hz, CH_2OH) 3.30–3.58 (2 H, dt as Ψq, $J_{NH,CH}$ 6.5, $J_{CH,CH}$ 6.1 Hz, CH_2NH), and 1.65–1.73 (2 H, tt as Ψp, $CH_2CH_2CH_2$).

Anal. Calcd for $C_{11}H_{15}NO_3$ (209.26): C, 63.13; H, 7.24; N, 6.70. Found: C, 62.46; H, 7.03; N, 6.52.

*Benzyl 3-(benzyloxycarbonylamino)propyl di-*N*-isopropylphosphoramidite.*—Dichloro(diisopropylamino)phosphine (7.08 g, 33.3 mmol) in 50 mL of tetrahydrofuran and 36 mL of diisopropylethylamine in a flask closed with a septum were cooled with an ice–salt mixture. Over a period of 30 min, a solution of 7.26 g (34.7 mmol) of 3-(benzyloxycarbonylamino)-1-propanol in 5 mL of tetrahydrofuran was added from a syringe. The mixture was stirred for 18 h at 25°, at which time 4 mL (38.3 mmol) of benzyl alcohol in 6 mL of tetrahydrofuran was added, and the mixture was stirred for an additional 4 h at 25°. The mixture was filtered, and the filtrate was diluted with 600 mL of acid-free EtOAc and extracted 3 times with 250-mL portions of 0.1 M phosphate buffer, pH 7.0. The organic layer was dried ($MgSO_4$) and evaporated to yield a viscous, slightly yellow liquid. This was fractionated by chromatography on 200 g of silica gel that had been activated at

110° overnight. The column was first eluted with 10:1 hexane–triethylamine to give dibenzyl di-*N*-isopropylphosphoramidite, then with 7:3:1 hexane–diethyl ether–triethylamine to give 4.94 g (11 mmol, 33%) of the desired benzyl 3-(benzyloxycarbonylamino)propyl di-*N*-isopropylphosphoramidite followed by bis[3-(benzyloxycarbonylamino)propyl] di-*N*-isopropylphosphoramidite.

^{1}H-NMR ($CDCl_3$) data for the title compound: δ 7.20–7.22 (10 H, m, Ar), 5.18–5.30 (1 H, br. s, N*H*), 5.04–5.13 (2 H, s, PhCH_2 of benzyl carbamate), 4.60–4.79 (2 H, m, PhCH_2OP), 3.68–3.80 (2 H, m, OCH_2 of propyl), 3.56–3.80 (2 H, m, C*H* of isopropyl), 3.24–3.40 (2 H, m, CH_2NH), 1.76–1.87 (2 H, tt as Ψp, $CH_2CH_2CH_2$), and 1.11–1.27 (12 H, 2 d, CH_3 of isopropyl); ^{31}P-NMR ($CDCl_3$, broadband decoupled): δ 146.9 (1 P, s).

*2,3,6-Tri-*O*-benzoyl-4,5-di-*O*-benzyl-1-*O*-(tetrahydro-2-pyranyl)-*D*-*myo*-inositol* (**2a**) *and 1,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-2-*O*-(tetrahydro-2-pyranyl)-*D*-*myo*-inositol* (**2b**).—A 3:1 mixture of 2,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol (**1a**) and 1,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol (**1b**) (10 g, 14.9 mmol) in 125 mL of dry toluene containing 100 mg of *p*-toluenesulfonic acid was treated with 3 mL of 3,4-dihydro-2*H*-pyran. After 18 h at 25°, HPLC analysis revealed the complete conversion of the reactants into less polar compounds. After the addition of 3 mL of triethylamine, the solution was extracted 3 times with water and dried ($MgSO_4$), and the solvent was evaporated. The syrupy residue was fractionated by preparative HPLC employing a step-gradient starting at 83:17 MeOH–water. Isomer **2a** was eluted first (6.42 g, 57%).

Anal. Calc. for $C_{46}H_{44}O_{10}$ (756.85): C, 73.00; H, 5.87. Found: C, 72.63; H, 6.08.

Isomer **2b** was obtained in a yield of 12% (1.34 g). Each of the two isomers **2a** and **2b** was a pair of diastereomers. Some fractions in the elution of **2a** contained the pure diastereomers and were used in separate NMR experiments to determine the ^{1}H chemical shifts listed here.

Compound **2a**, diastereomer A ($CDCl_3$): δ 7.08–8.12 (25 H, m, Ar), 6.04–6.07 (1 H, dd as Ψt, *J* 2.8 Hz, H-2), 5.93–5.99 (1 H, dd as Ψt, *J* 10.0 Hz, H-6), 5.45–5.49 (1 H, dd, $J_{2,3}$ 3.0, $J_{3,4}$ 10.2 Hz, H-3), 4.79–4.86 [3 H, m, PhCH_2 and C*H*(OCH_2-)O- of tetrahydropyranyl (Thp)], 4.65–4.74 (2 H, AB, PhCH_2), 4.26–4.31 (1 H, dd as Ψt, *J* 9.8 Hz, H-4 or H-5), 4.11–4.16 (1 H, dd, $J_{1,2}$ 3.0, $J_{1,6}$ 10.2 Hz, H-1), 3.81–3.90 (2 H, m overlapped by dd as Ψt, *J* 9.8 Hz, H-4 or H-5 and Thp-H), 3.49–3.54, and 1.20–1.54 (2 m, 1 H and 6 H, Thp-H).

Compound **2a**, diastereomer B ($CDCl_3$): δ 7.10–8.12 (25 H, m, Ar), 6.04–6.08 (1 H, dd as Ψt, *J* 2.9 Hz, H-2), 5.92–5.98 (1 H, dd as Ψt, *J* 10.0 Hz, H-6), 5.44–5.49 (1 H, dd, $J_{2,3}$ 2.8, $J_{3,4}$ 10.2 Hz, H-3), 4.95–4.98 [1 H, dd as Ψs, C*H*(OCH_2-)O- of Thp], 4.84–4.90 (2 H, AB, CH_2), 4.84–4.90 (2 H, AB, CH_2), 4.71–4.80 (2 H, AB, CH_2), 4.34–4.41 (1 H, dd as Ψt, *J* 9.9 Hz, H-4 or H-5), 4.22–4.27 (1 H, dd, $J_{1,2}$ 2.8, $J_{1,6}$ 10.0 Hz, H-1), 3.90–3.96 (1 H, dd as Ψt, *J* 9.7 Hz, H-4 or H-5), 3.56–3.64, 3.28–3.35, and 1.20–1.55 (3 m, 1 H, 1 H and 6 H, Thp-H).

Compound **2b**, 1:1 mixture of diastereomers ($CDCl_3$): δ 7.04–8.12 (50 H, m, Ar), 6.15–6.21 (1 H, dd as Ψt, *J* 10.1 Hz, H-6), 6.08–6.15 (1 H, dd as Ψt, *J* 10.1

Hz, H-6), 5.45–5.50 (1 H, dd, $J_{1(3),2}$ 2.6 Hz, $J_{1(3),6(4)}$ 10.0 Hz, H-1 or H-3), 5.43–5.48 (1 H, dd, $J_{1(3),2}$ 2.6 Hz, $J_{1(3),6(4)}$ 10.2 Hz, H-1 or H-3), 5.27–5.33 (1 H, dd, $J_{1(3),2}$ 2.8, $J_{1(3),6(4)}$ 10.2 Hz, H-1 or H-3), 5.21–5.27 (1 H, dd, $J_{1(3),2}$ 2.8, $J_{1(3),6(4)}$ 10.4 Hz, H-1 or H-3), 4.63–4.93 [12 H, m, CH_2, CH(OCH$_2$-)O- of Thp, and H-2], 4.41–4.48 (1 H, dd as Ψt, J 9.8 Hz, H-4 or H-5), 4.34–4.41 (1 H, dd as Ψt, J 9.8 Hz, H-4 or H-5), 3.88–3.95 (2 H, m, H-4 and H-5), 3.80–3.89, 3.70–3.79, 3.01–3.19, 2.92–2.99, and 1.25–2.22 (5 m, 1 H, 1 H, 1 H, 1 H, 12 H, Thp-H).

*2,3,6-Tri-*O*-benzoyl-4,5-*O*-(dibenzyloxyphosphinyl)-1-*O*-(tetrahydro-2-pyranyl)-*D*-myo-inositol* (**4**).—Compound **2a** (2.13 g, 2.81 mmol, mixture of diastereomers) was stirred in 70 mL of 95% EtOH with 4 g of Pearlman's catalyst under H_2 at 1 atm. The reaction, monitored by HPLC, was complete after 18 h. The catalyst was removed by filtration, the solvent was evaporated, and the product 2,3,6-tri-*O*-benzoyl-1-*O*-(tetrahydro-2-pyranyl)-D-*myo*-inositol (**3**) was used in the following reaction without further purification.

Compound **3** (1 g, 1.73 mmol), 2 g of 1*H*-tetrazole, and 3.5 mL of dibenzyl di-*N*-isopropylphosphoramidite in 30 mL of dry CH_2Cl_2 were stirred for 3.5 h at 25°. After the addition of another 1.5 mL of phosphitylating agent, the mixture was stirred for 4.5 h. The solution was cooled to −78°, 3.1 g of *m*-chloroperoxybenzoic acid was added, and the mixture was allowed to warm up to 25° overnight. Toluene (200 mL) was added, and the solution was extracted with 5% $NaHCO_3$, then water. The organic layer was dried ($MgSO_4$), the solvents were evaporated, and the residue in acetonitrile was fractionated by preparative HPLC using a step-gradient starting at 82:18 MeOH–water. Compound **4**, diastereomer A was eluted at 290 min with 84:16 MeOH–water, and diastereomer B was eluted at 360 min with 84:16 MeOH–water. The combined yield of syrup was 992.5 mg (880 μmol, 51%).

^{1}H-NMR ($CDCl_3$), diastereomer A: δ 6.74–8.12 (35 H, m, Ar), 6.05–6.13 (2 H, 2 overlapped dd as Ψt, $J_{1(3),2}$ 3.0, $J_{1(5),6}$ 10.1 Hz, H-2 and H-6), 5.52–5.58 (1 H, dd, $J_{2,3}$ 3.2, $J_{3,4}$ 10.0 Hz, H-3), 5.26–5.36 (1 H, ddd as Ψq, $J_{H,P}$ 9.6 Hz, $J_{H,H}$ 9.6 Hz, H-4 or H-5), 4.95–5.06 [3 H, m, PhCH_2OP and CH(OCH$_2$-)O- of Thp], 4.62–4.90 (5 H, m, PhCH_2OP and H-4 or H-5), 4.38–4.48 (2 H, m, PhCH_2OP), 4.16–4.22 (1 H, dd, $J_{1,2}$ 2.8, $J_{1,6}$ 10.2 Hz, H-1), 3.76–3.87, 3.45–3.55, and 0.80–1.60 (3 m, 1 H, 1 H, 6 H, Thp-H); ^{31}P-NMR ($CDCl_3$, broadband decoupled): δ −1.01 (1 P, s) and −1.56 (1 P, s).

^{1}H-NMR ($CDCl_3$), diastereomer B: δ 6.78–8.18 (35 H, m, Ar), 6.01–6.09 (2 H, 2 overlapped dd as Ψt, $J_{1(3),2}$ 3.0, $J_{1(5),6}$ 10.2 Hz, H-2 and H-6), 5.48–5.54 (1 H, dd, $J_{2,3}$ 3.0, $J_{3,4}$ 10.2 Hz, H-3), 5.34–5.43 (1 H, ddd as Ψq, $J_{H,P}$ 9.6, $J_{H,H}$ 9.6 Hz, H-4 or H-5), 4.97–5.11 (3 H, m, PhCH_2OP and H-4 or H-5), 4.92–4.96 [1 H, dd as Ψs, CH(OCH$_2$-)O- of Thp], 4.63–4.91 (4 H, m, PhCH_2OP), 4.38–4.48 (2 H, m, PhCH_2OP), 4.26–4.32 (1 H, dd, $J_{1,2}$ 2.8, $J_{1,6}$ 10.2 Hz, H-1), 3.44–3.55, 3.25–3.32, and 0.80–1.60 (3 m, 1 H, 1 H, 6 H, Thp-H); ^{31}P-NMR ($CDCl_3$, broadband decoupled): δ −0.99 (1 P, s) and −1.51 (1 P, s).

*2,3,6-Tri-*O*-benzoyl-4,5-bis-*O*-(dibenzyloxyphosphinyl)-*D*-myo-inositol* (**5**).—Compound **4** (992 mg) was dissolved in 40 mL of tetrahydrofuran, 80 mL of a 3:1

mixture of 88% formic acid and water was added, and the solution was stirred for 30 min at 25°. After this time, HPLC revealed complete conversion of the two diastereomers into one more polar compound. The mixture was poured into 1 L of water to precipitate the product in quantitative yield, $[\alpha]_{546}$ + 9.43° (95% EtOH); ^{1}H-NMR ($CDCl_3$): δ 6.75–8.18 (35 H, m, Ar), 6.01–6.06 (1 H, dd as Ψt, J 2.8 Hz, H-2), 5.88–5.96 (1 H, dd as Ψt, J 10.2 Hz, H-6), 5.49–5.55 (1 H, dd, $J_{2,3}$ 3.0, $J_{3,4}$ 10.2 Hz, H-3), 5.27–5.36 (1 H, ddd as Ψq, $J_{H,P}$ 9.3, $J_{H,H}$ 9.5 Hz, H-4 or H-5), 4.93–5.11 (3 H, m, PhCH_2OP and H-4 or H-5), 4.61–4.89 (4 H, m, PhCH_2OP), 4.36–4.47 (2 H, m, PhCH_2OP), and 4.14–4.20 (1 H, dd, $J_{1,2}$ 3.0, $J_{1,6}$ 10.2 Hz, H-1); ^{31}P-NMR ($CDCl_3$, broadband decoupled): δ −1.827 (1 P, s) and −2.347 (1 P, s); FABMS: m/z 1013 $(M + H)^+$.

*1-O-[(Benzyloxy)-3-(benzyloxycarbonylamino)propoxyphosphinyl]-2,3,6-tri-O-benzoyl-4,5-bis-O-(dibenzyloxyphosphinyl)-*D-myo-*inositol* (**6**).—Compound **5** (200 mg, 197 μmol), 176 mg (394 μmol) of benzyl 3-(benzyloxycarbonylamino)propyl di-*N*-isopropylphosphoramidite, and 100 mg of 1*H*-tetrazole in 10 mL of dry CH_2Cl_2 were stirred for 1 h at 25°, at which time HPLC revealed complete conversion into a more lipophilic compound. The mixture was cooled to −78°, treated with 500 mg of *m*-chloroperoxybenzoic acid, and allowed to warm up to 25° overnight. Toluene (200 mL) was added, the solution was extracted with 5% $NaHCO_3$ and water, and the organic layer was dried ($MgSO_4$). The solvents were evaporated and the residue was fractionated by preparative HPLC to yield 200 mg (74%) of product **6**, $[\alpha]_{546}$ −10.6° (95% EtOH); ^{1}H-NMR ($CDCl_3$): δ 6.75–8.15 (45 H, m, Ar), 6.16–6.20 (1 H, m, H-2), 6.06–6.16 (1 H, dd as Ψt, J 10.2 Hz, H-6), 5.51–5.57 (1 H, dd, $J_{2,3}$ 2.9, $J_{3,4}$ 10.0 Hz, H-3), 5.27–5.38 (1.5 H, m, H-4 or H-5 and N*H* of one diastereomer), 4.55–5.07 (14.5 H, m, PhCH_2, H-4 or H-5, H-1, and N*H* of one diastereomer), 3.80–3.92 (1 H, m, OCH_2 of propyl of one diastereomer), 3.55–3.96 (1 H, m, OCH_2 of propyl of one diastereomer), 3.02–3.10 (1 H, dt as Ψq, $J_{NH,CH}$ 6.2 Hz, CH_2NH of one diastereomer), 2.79–2.87 (1 H, dt as Ψq, $J_{NH,CH}$ 6.2 Hz, CH_2NH of one diastereomer), and 1.53–2.00 (2 H, br. m, $CH_2CH_2CH_2$); ^{31}P-NMR ($CDCl_3$, broadband decoupled): δ −1.445 (0.5 P, s, P-1 of diastereomer), −1.475 (0.5 P, s, P-1 of one diastereomer), −1.653 (1 P, s, P-4 or P-5), and −2.141 (1 P, s, P-4 or P-5).

*1-[3-Aminopropoxy(hydroxy)phosphinyl)]-2,3,6-tri-O-benzoyl-*D-myo-*inositol 4,5-bisphosphate* (**7**).—Compound **6** (198 mg, 144 μmol) in glacial acetic acid was hydrogenolyzed over 200 mg of Pearlman's catalyst for 6 h. The catalyst was removed and the filtrate was lyophilized to furnish pure **7** as the free acid in quantitative yield.

^{1}H-NMR (D_2O), ammonium salt: δ 7.28–8.17 (15 H, m, Ar), 6.09–6.16 (1 H, dd as Ψs, H-2), 5.78–5.88 (1 H, dd as Ψt, J 10.2 Hz, H-6), 5.41–5.50 (1 H, dd as Ψd, J 10.2 Hz, H-3), 4.89–5.00 (1 H, ddd as Ψq, $J_{H,P}$ 9.5 Hz, H-4 or H-5), 4.60–4.80 (2 H, m, H-1 and H-4 or H-5), 3.50–3.63 (2 H, m, $J_{NH,CH}$ 7.1 Hz, OCH_2 of propyl), 2.64–2.75 (2 H, t, J 7.1 Hz, CH_2NH), and 1.47–1.60 (2 H, d, J 7.0 Hz,

CH_2CH_2CH); ^{31}P-NMR (D_2O), ammonium salt: δ 0.170–0.114 (2 P, d, J 9.2 Hz, P-4 and P-5) and −0.660– − 0.528 (1 P, dt as Ψq, J_d 8.2, J_t 6.6 Hz, P-1).

1-[3-Aminopropoxy(hydroxy)phosphinyl]-D-myo-*inositol 4,5-bisphosphate* (**8**).—Compound **7** was kept in 0.8 M NaOH for 18 h at 25° to saponify the benzoyl ester groups. The product was treated with Dowex 50 (H^+ form), and adjusted to pH 6 with NaOH. The solution was applied to a DEAE Sephadex A-25 column (3 g of adsorbent, lithium form) and eluted in 5-mL fractions with a linear gradient of 0–500 mM LiCl in 100 mM LiOAc, pH 4.0. The three phosphate-containing peaks that were eluted were pooled separately and evaporated to dryness. Each residue was suspended in 10 mL of MeOH, 20 mL of acetone was added, and the precipitate was centrifuged. The liquid was decanted and the precipitation was repeated. The dry residue was dissolved in water and treated with Dowex 50 (H^+). The product was lyophilized as the free acid, or the pH was adjusted to 6 prior to lyophilization. Fractions 10–30 (50–175 mM LiCl) consisted of 11 mg (free acid) of an unknown phosphate compound that did not contain inositol, fractions 31–50 (175–290 mM LiCl) gave 43 mg of **8** (90.2 μmol, 63% as the free acid, according to a phosphate assay), and fractions 51–80 (290–470 mM LiCl) contained 21.3 mg of $InsP_3$ (as the free acid). The results suggest a 35% hydrolysis of the phosphate diester bond during saponification. Debenzoylation without phosphodiester cleavage was subsequently accomplished with concentrated ammonium hydroxide at 55° for 18 h. The product (**8**) had $[\alpha]_{546}$ −24.3° (water, pH 6.4). NMR spectra were determined on the sodium salt in D_2O at pH 6, and signal assignments were based on two-dimensional H-H and P-H spectra.

^{1}H-NMR: δ 4.24–4.33 (1 H, dd as Ψq, partially overlapping with H-2, $J_{H,P}$ 9.6, $J_{H,H}$ 9.6 Hz, H-4), 4.22–4.26 (1 H, dd as Ψt, partially overlapping with H-4, J 2.8 Hz, H-2), 3.96–4.13 (4 H, m, H-1, H-5, and OCH_2 of propyl), 3.87–3.95 (1 H, dd as Ψt, J 9.6 Hz, H-6), 3.69–3.75 (1 H, dd, $J_{2,3}$ 2.8, $J_{3,4}$ 9.6 Hz, H-3), 3.12–3.20 (2 H, t, J 7.2 Hz, CH_2NH), and 1.95–2.07 (2 H, tt as Ψp, $CH_2CH_2CH_2$); ^{31}P-NMR: δ 3.20–3.50 (1 P, d, J 9.2 Hz, P-4 or P-5), 2.60–2.88 (1 P, d, J 8.8 Hz, P-4 or P-5), 0.08–0.30 (1 P, dt as Ψq, J_d 8.6, J_t 5.6 Hz, P-1); FABMS (positive): m/z 478 $(M + H)^+$ and 500 $(M + Na)^+$.

1-O-[3-Formylaminopropoxy(hydroxy)phosphinyl]-D-myo-*inositol 4,5-bisphosphate* (**9**).—Compound **7** (120.6 mg of free acid, 144.7 μmol) in 1 mL of formamide and 5 mL of 1% methanolic NaOH was kept for 3 days at 25°. The mixture was diluted with 6 mL of water and treated with Dowex 50 (H^+) to remove the cations. The Dowex was removed and the filtrate was adjusted to pH 7 with NaOH. The water was evaporated and 10 mL of acetone was added to precipitate the product from the formamide solution. The precipitate was centrifuged, the supernatant was decanted, and the precipitate was redissolved in 1 mL of water and precipitated again with 10 mL of acetone to remove the formamide. The product was chromatographed on 3 g of DEAE Sephadex A-25 in 100 mM ammonium formate, pH 4.0, with a linear gradient from 0 to 500 mM LiCl (400 mL). Fractions of 5 mL were collected. The product was eluted between 200 and

400 mM LiCl in a yield of 71.4 mg (free acid, 98%), ^{1}H-NMR (sodium salt, D_2O, pH 7): δ 8.00–8.07 (1 H, s, C*H*O), 4.20–4.32 (2 H, m, H-2 and H-4), 3.84–4.07 (5 H, m, H-1, H-5, H-6, and OCH_2 of propyl), 3.66–3.75 (1 H, dd, $J_{2,3}$ 2.8, $J_{3,4}$ 9.6 Hz, H-3), 3.28–3.37 (2 H, t, J 6.8 Hz, CH_2NH), and 1.80–1.92 (2 H, tt as Ψp, $CH_2CH_2CH_2$); ^{31}P-NMR (sodium salt, D_2O, pH 7): δ 4.30–4.60 (1 P, d, J 8.4 Hz, P-4 or P-5), 3.65–4.05 (1 P, d as Ψs, P-4 or P-5), and 0.30–0.55 (1 P, dt as Ψq, J_d 8.6, J_t 5.6 Hz, P-1); FABMS (negative, free acid): m/z 504 $(M - H)^-$.

Coupling of 8 to carbonyldiimidazole-activated agarose (Reacti-Gel).—An ice-cold solution of the sodium salt of **8** (4.2 mg as the free acid) in 1 mL of water, pH 6, was added to 1 mL of ice-cold 1 M carbonate buffer, pH 10.0. Cooled Reacti-Gel slurry in acetone, corresponding to 1 mL of settled gel, was filtered to remove of the solvent, and the wet gel was added to the inositol phosphate solution. The mixture was slowly agitated on a shaker at 5° for 15 h and then filtered on a Büchner funnel, and the gel was washed with water and 0.9% NaCl containing 0.01% sodium azide. The product **10** was stored as a slurry in this solution at 5° until it was used. The coupling, based on phosphate analysis, was 40%, which corresponded to 2.3 mg or 4.8 μmol of ligand per mL of gel.

Coupling of 8 to 4-azido-2-hydroxybenzoic acid.—The sodium salt of **8** (21 mg calculated as the free acid, 44 μmol) was dissolved in 1.8 mL of 0.5 M freshly prepared $NaHCO_3$. Succinimido 4-azido-2-hydroxybenzoate (47.6 mg, 175 μmol) in 1 mL of acetonitrile was added under the dim light of a darkroom lamp, and the mixture was kept in the dark for 5 days. The solution was diluted with 3 mL of 100 mM LiOAc buffer, pH 4.0, filtered to remove some solid precipitate, and applied to a DEAE Sephadex A-25 column (chloride form, prepared from 3 g of adsorbent). The ion-exchanger was eluted with a linear gradient of 0–500 mM LiCl in 100 mM LiOAc, pH 4.0, in fractions of 5 mL. Two phosphate-containing peaks were obtained, a minor one containing unreacted **8** and eluted at 150–270 mM LiCl and the major peak corresponding to the coupled product at 270–500 mM LiCl. The fractions comprising the latter peak were evaporated to dryness, the solid residue was taken up in 1:2 MeOH–acetone, and the suspension was centrifuged to give the product as a solid. This extraction was repeated once to remove the LiCl and LiOAc and yielded 21.8 mg (57% by phosphate assay) of 1-*O*-[3-(4-azido-2-hydroxybenzoylamino)propoxy(hydroxy)phosphinyl]-D-*myo*-inositol 4,5-bisphosphate (**11**), ^{1}H-NMR (D_2O): δ 7.72–7.80 (1 H, d, J 8.4 Hz, arom. H-6), 6.50–6.59 (2 H, s and d partially overlapping, arom. H-3 and H-5), 4.17–4.29 (2 H, H-4 and H-2), 3.98–4.09 (3 H, m, H-1 and OCH_2 of propyl), 3.89–3.98 (2 H, H-5 and H-6), 3.67–3.74 (1 H, dd, H-3), 3.45–3.54 (2 H, t, CH_2NH), and 1.92–2.01 (2 H, tt as Ψp, $CH_2CH_2CH_2$); ^{31}P-NMR (D_2O): δ 4.4–5.1 (1 P, broad s, P-4 or P-5), 3.7–4.4 (1 P, broad s, P-4 or P-5), and 0.2–0.7 (1 P, s, P-1).

*1-O-[3-(4-azido-2-hydroxy-3,5-diiodobenzoylamino)propoxy(hydroxy)phosphinyl]-*D-myo-*inositol 4,5-bisphosphate* (**12**).—Seventy Iodo-Beads (Pierce) were washed with 50 mL of water and 50 mL of LiOAc buffer and partially dried on filter paper. The beads were added to 12.6 μmol of compound **11** (corresponding to 8 mg of the

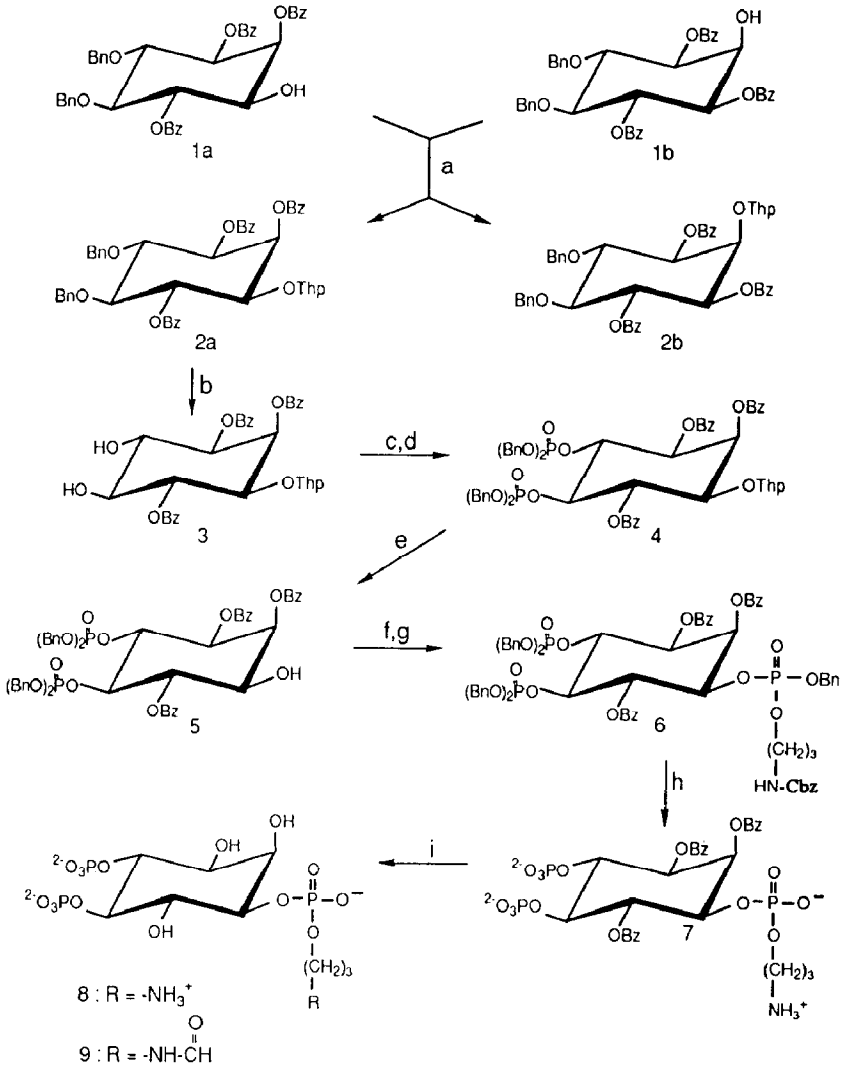
free acid) in 4 mL of 100 mM LiOAc buffer, pH 5.5. Sodium iodide (20.7 mg, 138 μmol) was added, and the mixture was agitated on a rotary shaker at 25° for 1 h. The liquid was removed by pipet, the beads were washed twice with 4 mL of the buffer, and the combined solution was passed over a Dowex 50 (H^+)column. The acidic eluate was adjusted to pH 7 with a LiOH solution, the mixture was lyophilized, and the dry residue was suspended in 3 mL of MeOH and transferred into a centrifuge tube. Acetone (9 mL) was added and the precipitate was centrifuged. The liquid was decanted, the precipitate was resuspended in 3 mL of MeOH with sonication, 9 mL of acetone was added, and the tube was centrifuged again. The liquid was decanted, and the precipitate was dissolved in water and lyophilized to give the pure product (**12**), UV (H_2O): λ_{max} 350 nm; ^{1}H-NMR (D_2O): δ 8.23–8.29 (1 H, s, arom. H-6), 4.19–4.30 (2 H, H-4, and H-2), 3.90–4.10 (5 H, m, H-1, H-5, H-6, and OCH_2 of propyl), 3.68–3.74 (1 H, dd as Ψd, H-3), 3.48–3.54 (2 H, t, CH_2NH), and 1.95 (2 H, $CH_2CH_2CH_2$); ^{31}P-NMR (D_2O): δ 4.3–4.7 (1 P, s, P-4 or P-5), 3.7–4.1 (1 P, s, P-4 or P-5), and 0.3–0.6 (1 P, dt as Ψd, P-1).

Radioactive **12**.—Forty Iodo-Beads were washed as above and added to 6.28 μmol of **11** (corresponding to 4 mg of the free acid) in 1.5 mL of 100 mM LiOAc buffer, pH 5.5. One mL of LiOAc buffer containing 1 mCi of $^{125}I^-$ was added, and the mixture was shaken on a rotary shaker at 25° for 30 min, when 10.4 mg (69 μmol) of NaI in 200 μL of LiOAc was added to quench the radioactive iodination. After the mixture was shaken for another hour, the liquid was transferred by pipet into a plastic centrifuge tube, the beads were washed twice with 2-mL portions of LiOAc buffer, and the combined solution was dried under a stream of N_2. The dry residue was suspended in 2 mL of MeOH with sonication, 6 mL of acetone was added, and the precipitate was centrifuged. The liquid was decanted, and the solid precipitate was dissolved in 1 mL of water and transferred to a tube containing 30 mL of acetone. The precipitate was centrifuged, the supernatant was decanted, and the solid was dried in the dark. The specific radioactivity of the [^{125}I]-**12** was 31.6 mCi/mmol, which indicates that about 20% of the radioactivity was incorporated into the product.

RESULTS AND DISCUSSION

Our synthesis started with the mixture of 2,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol (**1a**) and 1,3,6-tri-*O*-benzoyl-4,5-di-*O*-benzyl-D-*myo*-inositol (**1b**) (Scheme 1) that can be obtained in good yield by inversion of the hydroxyl group at C-6 of 1,2,5-tri-*O*-benzoyl-3,4-di-*O*-benzyl-D-*chiro*-inositol via its trifluoromethanesulfonate ester[6]. Isomers **1a** and **1b** are separable by preparative HPLC, but the resolution is improved if the free hydroxyl groups are first protected.

The protecting group at position 1 of **2a** had to meet a number of requirements: it had to be introduced under conditions that avoided cleavage or migration of benzoyl esters, it had to be stable during hydrogenolysis of the benzyl ether groups



Scheme 1. Steps in the synthesis of compounds **8** and **9**. The reagents are: *a*, 3,4-dihydro-2-*H*-pyran and *p*-toluenesulfonic acid in toluene; *b*, H_2 and $Pd(OH)_2$–C in ethanol; *c*, dibenzyl di-*N*-isopropylphosphoramidite and 1*H*-tetrazole in CH_2Cl_2; *d*, *m*-chloroperoxybenzoic acid; *e*, 2:3:1 tetrahydrofuran–88% formic acid–water; *f*, benzyl 3-(benzyloxycarbonylamino)propyl di-*N*-isopropylphosphoramidite and 1*H*-tetrazole in CH_2Cl_2; g, *m*-chloroperoxybenzoic acid; *h*, H_2 and $Pd(OH)_2$–C in ethanol; *i*, 0.8 M NaOH or concentrated NH_4OH for **8** and methanolic ammonia in formamide for **9**. Bn = benzyl, Bz = benzoyl, Cbz = benzyloxycarbonyl, Thp = tetrahydro-2-pyranyl.

and during the phosphorylation reaction, and it had to be removable under conditions that would not affect the labile dibenzyl phosphate triester. Although the *tert*-butyldimethylsilyl and isopropyldimethylsilyl groups could be introduced smoothly and in good yield[13,14], the isopropyldimethylsilyl group proved to be unstable during the following steps whereas the *tert*-butyldimethylsilyl group was too stable. Acidic conditions that did not affect the phosphotriester bonds, like

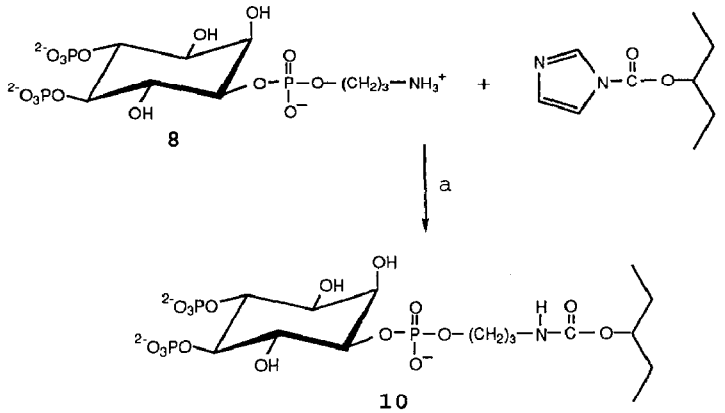
88% formic acid at 25°, did not cleave the *tert*-butyldimethylsilyl group. The use of quaternary ammonium fluorides or HF in acetonitrile, commonly employed for the deprotection of silylated compounds[15], led to inseparable mixtures, most likely due to partial cleavage of the benzyl phosphate ester bonds. Eventually, the tetrahydro-2-pyranyl group was found to be satisfactory for our purpose. Because the inositol compounds are chiral and the tetrahydro-2-pyranyl group contains a chiral carbon, however, **2a** and **2b** each consist of a pair of diastereomers. Some fractions from the preparative HPLC purification of **2a** contained the pure separated diastereomers, and the NMR chemical shifts, which are somewhat different, have been assigned separately. The use of the nonchiral 4-methoxytetrahydro-4-pyranyl group as an alternative to the tetrahydro-2-pyranyl group[16] gave poor yields, whereas the methoxyethoxymethyl group was too stable towards acidic cleavage.

Hydrogenolysis of the benzyl groups of **2a** in ethanol, without cleaving the tetrahydropyranyl group, was accomplished by using a large excess of the highly active palladium hydroxide-on-carbon (Pearlman's catalyst). Hydrogenolysis with H_2 and palladium-on-charcoal proceeded at a reasonable rate only in an acidic solvent (acetic acid), which led to some loss of the tetrahydropyranyl group. Alternative procedures, such as a catalytic transfer hydrogenation with ammonium formate and palladium-on-charcoal[17] in methanol, led to benzoyl group migration, caused by ammonia resulting from formate oxidation; whereas a method that employs cyclohexene as the hydrogen donor and palladium hydroxide as catalyst[18] was unsuccessful in our hands.

Phosphorylation of the free vicinal hydroxyl groups of **3** was done by the phosphoramidite method[19] that we used previously[6,12]. Again, it was possible to separate the diastereomers by HPLC and assign the NMR signals to the two isomers. The tetrahydropyranyl group of **4** was removed smoothly and without phosphotriester cleavage by treatment with formic acid at 25° to yield one homogenous product from the mixture of diastereomers.

For the introduction into position 1 of a phosphodiester function having an extender arm and a terminal amino group, we prepared a new, asymmetric phosphite reagent by reacting dichloro(diisopropylamino)phosphine with one equivalent each of 3-(benzyloxycarbonylamino)-1-propanol and benzyl alcohol. Reaction with this phosphite reagent, followed by oxidation, gave the fully protected $InsP_3$ analogue **6**. Because the phosphorus at position 1 is chiral, **6** also consists of a pair of diastereomers. Although the isomers did not separate on HPLC, the observed heterogeneity of the ^{1}H-NMR spectrum and of the ^{31}P-NMR signal for the 1-phosphate confirmed that **6** was a mixture.

Deprotection of **6** was done in two steps. First the benzyl groups were removed by hydrogenolysis, whereupon the NMR spectrum became simpler in a manner consistent with loss of chirality at the 1-phosphate group. Treatment with 0.8 M NaOH for 18 h then saponified the benzoyl groups, unfortunately accompanied by 35% of phosphate diester cleavage. Attempts to cleave the benzoyl ester groups by ammonia in methanol or 1% NaOH in methanol to reduce diester cleavage gave



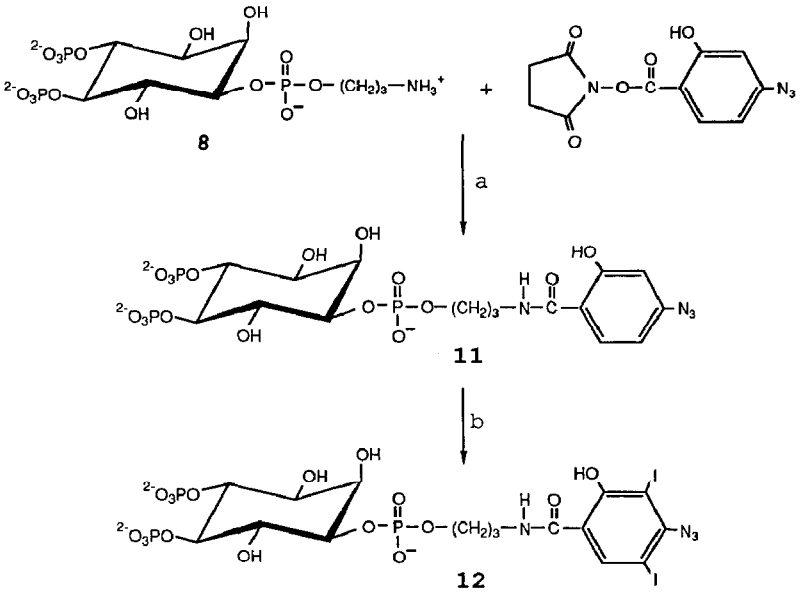
Scheme 2. Coupling of **8** to agarose beads. The conditions are: *a*, carbonate buffer, pH 10.0, 5°, 15 h.

incomplete debenzoylation because the material precipitated, whereas the addition of formamide to prevent precipitation caused formylation of the free amine. The resulting amide **9** proved to be a useful model compound in the calcium ion-release studies. Debenzoylation without phosphodiester cleavage was subsequently accomplished with concentrated ammonia at 55° for 18 h.

Compound **8** was coupled in a yield of 40% to carbonyldiimidazole-activated agarose (Scheme 2), where the $InsP_3$ is bound as the carbamate. This product is more stable than that formed with a cyanogen bromide-activated matrix, and the neutral linking group has no tendency to form an internal salt with the attached $InsP_3$. Reaction of **8** with succinimido 4-azido-2-hydroxybenzoate (Scheme 3) gave the photoaffinity analogue **11**, which was iodinated with both ^{125}I and ^{127}I by immobilized chloramine-T. The NMR spectrum of the nonradioactive product confirmed, in agreement with our earlier finding[5], that diiodination had occurred, at positions 3 and 5 of the aromatic ring. The λ_{max} for the diiodinated derivative is 350 nm, which allows affinity labeling at a wavelength that causes minimal damage to proteins or cells.

The calcium ion-releasing activity of the synthesized compounds was determined in a system employing saponin-permeabilized rat basophilic leukemia cells, with fluo-3 (Molecular Probes) as the Ca^{2+} indicator (Fig. 1, Table I). Compound **8** shows the lowest activity, with an EC_{50} value of 6.3 μM, which is similar to that for D-*myo*-2,4,5-$InsP_3$ (EC_{50} = 4.3 μM)[11]. This low activity may be due to the formation of an intramolecular salt between the terminal protonated amino group and one of the phosphates, or to the positive charge alone, because the uncharged $InsP_3$-analogue **9** has a higher activity (EC_{50} = 1.4 μM). The activity of **9** indicates that the affinity matrix should be an effective reagent for the purification of $InsP_3$-binding proteins.

Surprisingly high, in comparison to these structures, are the activities of the photoaffinity reagent **11** and, especially, its iodinated product **12**. The high activity



Scheme 3. Synthesis of the photoactivable derivatizing agent. The reagents are: *a*, 9:5 0.5 M sodium hydrogencarbonate–acetonitrile, 25°, 5 days in the dark; *b*, 0.1 M lithium acetate, pH 5.5, Iodo-Beads, NaI (^{125}I for radioactive **12**), 1 h, 25°.

may be related to the lipophilic character of the aromatic group in **11** and **12**, which is greater for the iodinated form. This aromatic structure could increase nonspecific lipophilic interaction and lead to a higher apparent activity. The aromatic group seems also to interact with other components of the cell, because light-induced reaction labels other proteins in addition to the $InsP_3$ receptor[7], an observation also made with other, similar derivatizing reagents[2]. In addition, stimulation of Ca^{2+} release by **12** lasts for a shorter time than that by $InsP_3$ (Fig.

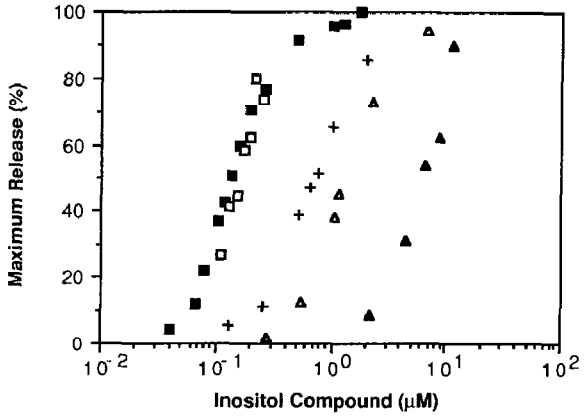


Fig. 1. Calcium ion-release curves of IP_3 and analogues. The symbols are: ■, $InsP_3$; □, **12**; +, **11**; △, **9**; ▲, **8**.

TABLE I

Ca^{2+}-Releasing activities of $InsP_3$ and derivatives

Compound	EC_{50} (μM)
$InsP_3$	0.173
8	6.3
9	1.4
11	0.72
12	0.26

2), indicating that **12** is inactivated more rapidly, either by its metabolism or by binding to membrane lipid, and the Ca^{2+} reuptake is almost as fast as if no inositol phosphate were present (Fig. 2). In view of this result, it is important for a successful labeling of the receptor protein that the photoactivation be carried out within seconds after binding of the reagent to the receptor, at a time when the Ca^{2+} release is still maximal.

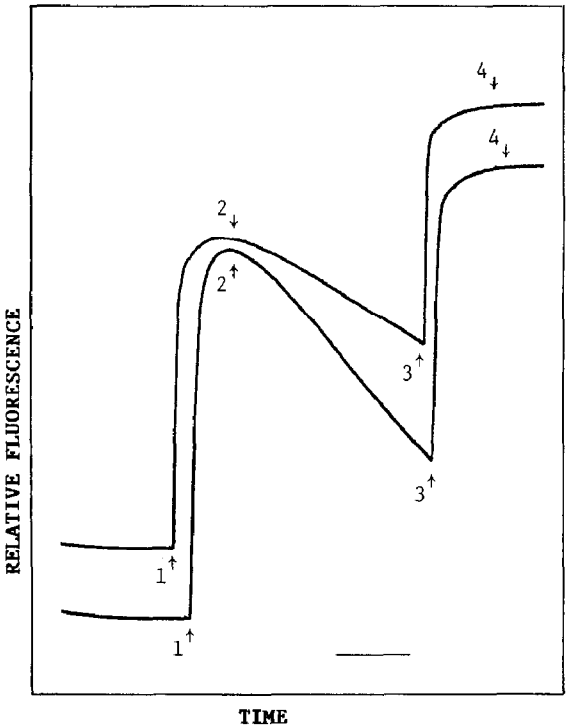


Fig. 2. Representative Ca^{2+}-release curves. *Top curve*, $InsP_3$; *bottom curve*, compound **12**, equimolar amount. Compounds were added at *arrow 1*, and the fluorescence was measured at *arrow 2*. Saturating amounts of $InsP_3$ were added at *arrow 3*, and the maximum response was measured at *arrow 4*. The *bar* indicates an elapsed time of 1 min.

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