Tethered IP_3. Synthesis and Biochemical Applications of the 1-*O*-(3-Aminopropyl) Ester of Inositol 1,4,5-Trisphosphate

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Abstract: A phosphodiester analogue of the second messenger Ins(1,4,5)P_3 has been synthesized and used to prepare a novel photoaffinity label and a selective bioaffinity matrix. A selectively protected inositol precursor was first converted by phosphite ester chemistry to an N-protected 1-*O*-(3-aminopropyl-1-phospho)-DL-*myo*-inositol and then phosphorylated to give a fully benzylated IP_3 derivative. Hydrogenolysis gives the title compound, which was converted to a photolabile analogue and was immobilized on a polymeric resin. The analogues all competed with [^{3}H]Ins(1,4,5)P_3 for binding to purified IP_3 receptors from rat brain. Reconstituted receptor liposomes showed calcium release when stimulated by the tethered IP_3 materials. None of the new materials were substrates for the 5-phosphatase or the 3-kinase that normally act on Ins(1,4,5)P_3.

Receptor-activated cleavage of phosphatidylinositol 4,5-bisphosphate releases the second messenger D-*myo*-inositol 1,4,5-trisphosphate Ins(1,4,5)P_3);[1] in turn, Ins(1,4,5)P_3 interacts stereospecifically with membrane receptors in a variety of cells to promote the release of Ca^{2+} from intracellular stores.[2] Several receptor proteins that specifically recognize Ins(1,4,5)P_3 and mediate its role in calcium release have been characterized very recently.[3] In addition, metabolism of Ins(1,4,5)P_3 by a 3-kinase and a 5-phosphatase begins a cascade of transformations leading to a host of inositol phosphates.[4] Despite the rapid biochemical progress, only a few reports describe the use of reactive analogues and resin-immobilized analogues of Ins(1,4,5)P_3 for protein characterization.[5,6] We now report the regioselective synthesis of the C-1 phosphodiester derivative of Ins(1,4,5)P_3 bearing a reactive aminopropyl tether (**1a**; Scheme I). This analogue and its photolabile derivative (**1b**) showed receptor binding and activation of Ca^{2+} release in vitro. Furthermore, the tethered derivative **1a** was also converted to affinity matrix **1c**, which was employed for the rapid purification of the Ins(1,4,5)P_3 receptor protein from rat brain.

Results and Discussion

We initially selected the 1-*O*-phosphate for modification as a phosphodiester on the basis of (a) the biological activity of semisynthetic 1-*O*-phosphodiester analogues[7] and the P-1 "caged" Ins(1,4,5)P_3 analogue,[8] (b) the versatile chemistry possible with a common protected inositol precursor, and (c) the ease of performing the multiple phosphorylations required for selective introduction of the linker arm. In addition, the low affinity of the unnatural L-*myo*-Ins(1,4,5)P_3 enantiomer for receptor binding[2a] obviated the need to use an enantiomerically pure ligand for initial experiments. Phosphite triester chemistry was selected to minimize problems arising from steric hindrance and to maximize yields. Thus, the novel activated phosphite **2** bearing a protected aminopropyl group was prepared from (benzyloxy)dichlorophosphine[9] in 60% yield by sequential reaction with diisopropylamine followed by *N*-Cbz-3-amino-1-propanol. Phosphitylation of the selectively protected inositol[10] **3** with reagent **2** in the presence of tetrazole in CH_2Cl_2 followed by oxidation with *m*-chloroperbenzoic acid gave the phosphate triester **4** in 88% yield (Scheme II). Deprotection (0.1 N HCl, H_2O–CH_3OH, 40 °C) followed by 4,5-bisphosphitylation and *m*-CPBA oxidation gave the fully benzylated 1-*O*-(3-aminopropyl) ester of Ins(1,4,5)P_3 (**5**) in racemic form in 80% yield. Hydrogenolysis (3 atm H_2, 10% Pd–C, ethanol) followed by ion-exchange chromatography (Chelex, sodium form) afforded the desired tethered Ins(1,4,5)P_3 (**1a**) in 75% yield.

Table I. In Vitro Biochemical Activity of Ins(1,4,5)P_3 Analogues **1a** and **1b**[a]

compd	K_i, nM	Ca release (ED_{50}), nM	substrate for 5-phosphatase	substrate for 3-kinase
Ins(1,4,5)P_3	10	50	yes	yes
1a	304	420	no	no
1b	236	375	no	no

[a] K_i values were determined by competition of compounds with [^{3}H]Ins(1,4,5)P_3 binding to washed rat cerebellar membranes.[12] ED_{50} values for calcium release were measured in $^{45}Ca^{2+}$ flux assays using purified rat cerebellar Ins(1,4,5)P_3 receptors reconstituted into lipid vesicles.[13] Sensitivity of the compounds as Ins(1,4,5)P_3 5-phosphatase and 3-kinase substrates was evaluated as described previously.[14] Data are mean values of assays performed in triplicate.

Table II. Comparison of Receptor Purification Strategies by Classical Means and by Using Immobilized Ins(1,4,5)P_3 (1c)[a]

fraction	specific activity, pmol/mg	fold purifcn	% yield
washed membranes	0.30	1	100
1% Triton X-100 solubilization	0.42	1.4	96
heparin–agarose	24	80	<7
concanavalin A–Sepharose	209	697	35
or			
aminopropyl Ins(1,4,5)P_3 affinity resin	230	767	29

[a] Ins(1,4,5)P_3 receptor preparation was obtained from rat cerebellum.[2c] The heparin–agarose column eluate fraction was divided into two equal portions; half was applied to concanavalin A–Sepharose, and half was applied to affinity resin **1c**. The data shown are representative of three replicate experiments.

The ^{31}P NMR of this product clearly showed three peaks in a 1:1:1 ratio: δ 5.58 (d, J = 7.8 Hz), 5.51 (d, J = 7.8 Hz) and 0.49 ppm

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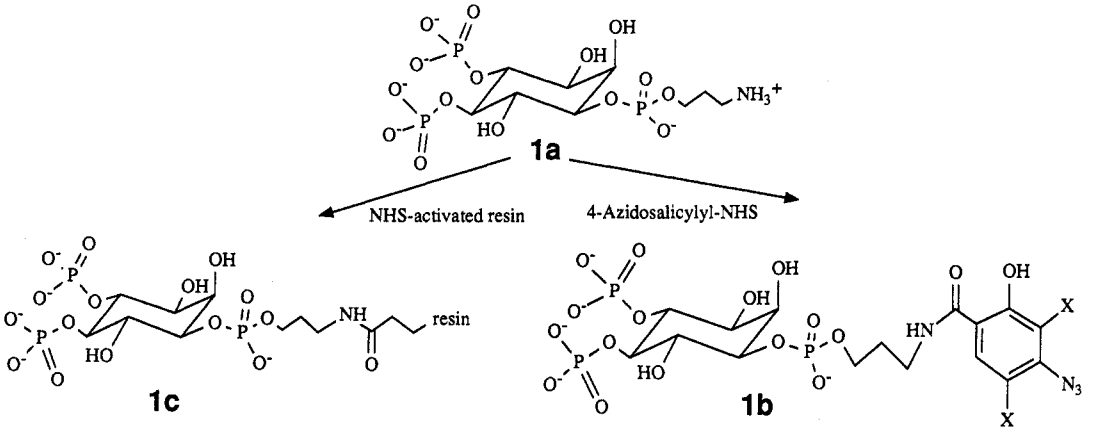
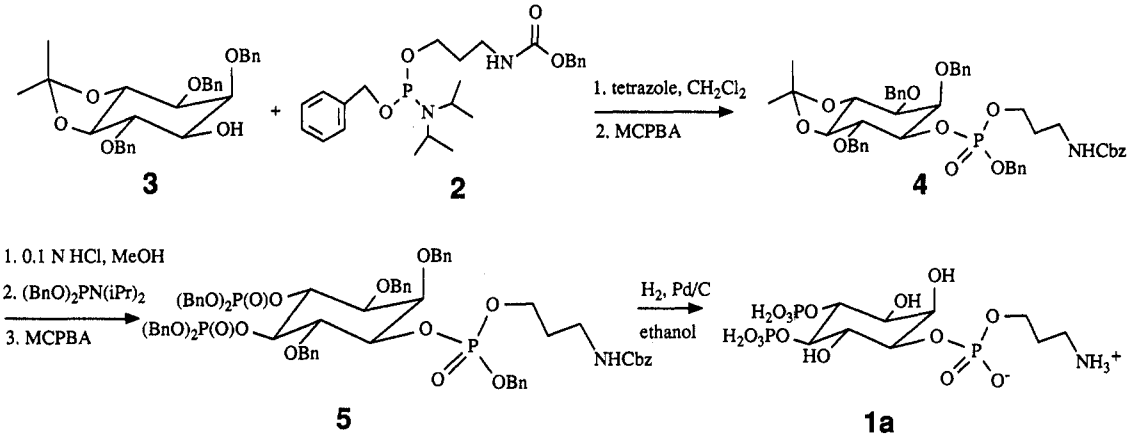
Scheme I. Design of Tethered Ins(1,4,5)P_3 and Associated Receptor-Targeted Probes (X = H, ^{125}I)



Scheme II. Synthesis of the 1-*O*-(3-Aminopropyl) Ester of Ins(1,4,5)P_3



(m) relative to external 85% phosphoric acid.

Reaction with *N*-hydroxysuccinimido 4-azidosalicylate[11] in aqueous DMF–0.25 M triethylammonium bicarbonate buffer in the dark gave, after purification on DEAE-cellulose, the 4-azidosalicylamide (ASA) derivative **1b**; this product combines photolability and a radioiodination site in the same moiety. Alternatively, the aminopropyl-tethered Ins(1,4,5)P_3 was immobilized on a solid support by reaction with a suspension of a resin-immobilized *N*-hydroxysuccinimide ester (Affi-Gel 10) in aqueous bicarbonate buffer (0 °C for 24 h).

Table I summarizes the in vitro biochemical activities observed for the parent aminopropyl derivative **1a** and the photoaffinity label **1b**. First, both aminopropyl Ins(1,4,5)P_3 **1a** and the ASA derivative **1b** displaced [^{3}H]-D-*myo*-Ins(1,4,5)P_3[12] bound to purified rat brain Ins(1,4,5)P_3 receptors.[2c] Second, both analogues showed activation of Ca^{2+} release from liposome-reconstituted receptor preparations.[13] Third, the two compounds derived from structure **1** were neither substrates nor inhibitors for the $InsP_3$ 5-phosphatase or the 3-kinase activities.[14]

Table II demonstrates the use of the resin-immobilized Ins(1,4,5)P_3 (**1c**) for purification of receptors for Ins(1,4,5)P_3. The eluate from the affinity column shows a single protein band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining, indicating a homogeneous preparation. The aminopropyl Ins(1,4,5)P_3 resin thus provides purified Ins(1,4,5)P_3 receptor with a slightly greater activity than can be obtained by other techniques.[2c,5] For example, Hirata et al.[5] reported 5–200-fold purifications for receptor binding, kinase, and phosphatase activity on Sepharose 4B immobilized Ins(1,4,5)P_3, in which linkers are coupled to the C-2 hydroxyl group through an ester linkage.

While the capacity of resin **1c** may be limited, reasonable quantities of highly purified receptor protein can be rapidly obtained with this material. In addition, this highly selective resin can be used to isolate Ins(1,4,5)P_3 receptors from low-abundance tissues and to distinguish Ins(1,4,5)P_3 receptors from Ins(1,3,4,5)P_4

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and Ins(1,2,3,4,5,6)P_6 receptors. Future studies will be directed at optimization of tether length, purification of other inositol polyphosphate receptors, and characterization of binding sites by photoaffinity labeling.

Experimental Methods

General Procedures (Synthesis). Methylene chloride was distilled from P_2O_5 and stored over 4-Å molecular sieves. Tetrahydrofuran was distilled from the sodium ketyl of benzophenone. *N,N'*-Dimethylformamide was dried by storing over 4-Å molecular sieves. Solvents were removed under reduced pressure (30 mmHg) or in vacuo at temperatures below 30 °C.

Column chromatography was performed with Merck silica gel 60 (230–400 mesh) using gravity elution. TLC was performed using Macherey Nagel silica gel G plates. Visualization was with UV light or by dipping in 10% phosphomolybdic acid in ethanol followed by heating.

^{31}P NMR spectra were taken on a GE-Nicolet NT-300 spectrometer at 121.5 MHz. Unless noted, they were run with broad-band proton decoupling.

(Benzyloxy)dichlorophosphine was prepared[9] from phosphorus trichloride and benzyl alcohol in 64% yield, bp 79 °C (0.75 mmHg). The compound was unstable even at −20 °C and could only be stored about 1 or 2 months.

Chloro(*N,N*-diisopropylamino)(benzyloxy)phosphine. A solution of diisopropylamine (3.64 g, 0.036 mol, 5.0 mL) in 5 mL of methylene chloride was added dropwise over 30 min to a stirred solution of (benzyloxy)dichlorophosphine (3.9 g, 0.018 mol) in 10 mL of methylene chloride at −20 °C. The mixture was allowed to warm to room temperature over 1 h and then stirred at room temperature for 30 min. Diisopropylammonium chloride was removed by filtration and washed with 2 × 3 mL of methylene chloride. The combined filtrates were evaporated, the resulting yellow oil was dissolved in 25 mL of dry ether, and the additional ammonium salt that precipitated was removed by filtration. Evaporation of the ether yielded the salt-free phosphine product: ^{31}P NMR ($CDCl_3$) δ 180.4 ppm; ^1H NMR ($CDCl_3$) δ 7.2–7.3 (5 H, m), 4.87 (2 H, t), 3.80 (2 H, m), 1.24 (6 H, d), 1.21 (6 H, d). The crude product was used without further purification.

(Benzyloxy)[(*N*-Cbz-3-aminopropyl)oxy](*N,N*-diisopropylamino)-phosphine (2). *N*-Cbz-3-amino-1-propanol (1.42 g, 6.8 mmol) was dissolved in 15 mL of methylene chloride, and diisopropylethylamine (1.76 g, 13.6 mmol) was added. The stirred solution was cooled to 0 °C under N_2 and a solution of chloro(*N,N*-diisopropylamino)(benzyloxy)phosphine (1.85 g, 6.8 mmol) in 10 mL of methylene chloride added dropwise over 3 min. The solution was stirred at 0 °C for 10 min then at room temperature for 75 min. The solution was diluted with another 50 mL of methylene chloride, washed (2 × 25 mL of 10% aqueous Na_2CO_3; 25 mL water), dried ($MgSO_4$), filtered, and concentrated. The crude oil remaining was chromatographed on silica gel by using ethyl acetate–hexane–triethylamine (20:20:1) as the eluting solvent to give 2.10 g (70%) of reagent **2** as a colorless oil: ^{31}P NMR ($CDCl_3$) δ 146 ppm; ^1H NMR ($CDCl_3$) δ 7.2–7.4 (10 H, m), 5.17 (1 H, br s), 5.02 (2 H, s), 4.63 (2 H, m), 3.65 (2 H, m), 3.58 (2 H, m), 3.24 (2 H, m), 1.75 (2 H, m), 1.12 (6 H, d), 1.11 (6 H, d).

Benzyl *N*-Cbz-3-amino-1-propyl 1-(2,3,6-Tri-*O*-benzyl-4,5-*O*-isopropylidene-*myo*-inosityl) Phosphate (4). A solution of 1,2,4-tri-*O*-benzyl-5,6-*O*-isopropylidene-*myo*-inositol (**3**;[10a] 450 mg, 0.9 mmol) in 20 mL of methylene chloride and (1-*H*)tetrazole (250 mg, 3.6 mmol) was stirred at room temperature while a solution of (benzyloxy)[(*N*-Cbz-3-amino-1-propyl)oxy](*N,N*-diisopropylamino)phosphine (630 mg, 1.4 mmol) in 3 mL of methylene chloride was added in one portion. The mixture was stirred at room temperature for 5 h and then cooled to −40 °C, and *m*-chloroperbenzoic acid (500 mg) added. The −40 °C bath was replaced with an ice bath and stirring was continued for 30 min at 0 °C and then for 30 min at room temperature. The mixture was diluted to 80 mL with methylene chloride, washed (2 × 10 mL of 10% aqueous Na_2SO_3; 2 × 10 mL of saturated $NaHCO_3$; 15 mL of water), dried ($MgSO_4$), filtered, concentrated, and chromatographed on silica gel by using ethyl acetate–hexane–triethylamine (50:50:1) to give 630 mg (80%) of phosphodiester **4** as a very viscous colorless oil: the ^{31}P NMR spectrum showed two peaks (two diastereomers present) δ 2.05 and 2.34 ppm; ^1H NMR ($CDCl_3$) δ 7.1–7.4 (25 H, m), 5.00 (2 H, s), 4.5–4.95 (10 H, m), 3.8–4.25 (4 H, m), 3.55 (1 H, t), 3.32 (1 H, q), 3.00 (2 H, m), 1.52 (2 H, m), 1.41 (3 H, s), 1.40 (3 H, s). MS (FAB) *m/q* 852.4 (M + 1).

Benzyl *N*-Cbz-3-amino-1-propyl 1-(2,3,6-Tri-*O*-benzyl-*myo*-inositol) Phosphate. Benzyl *N*-Cbz-3-amino-1-propyl 1-(2,3,6-tri-*O*-benzyl-4,5-*O*-isopropylidene-myo-inositol) phosphate (400 mg, 0.44 mmol) was dissolved in 9 mL of methanol and placed in an oil bath preheated to 40 °C. After a few minutes, 1 mL of 1 M hydrochloric acid was added and the solution stirred at 40 °C for 5 min. The solution was cooled in an ice bath and checked by TLC for complete reaction (silica gel, ethyl acetate–hexane 1:1). The acid was neutralized with excess sodium bicarbonate (0.30 g) and the solvent removed in vacuo at room temperature. The residue was stirred with 20 mL of methylene chloride for 20 min and then filtered and the solid washed with 10 mL of methylene chloride. The combined filtrates were evaporated to give 0.36 g of a colorless gum, which was used immediately in the next step without further purification.

Benzyl *N*-Cbz-3-amino-1-propyl 1-[2,3,6-Tri-*O*-benzyl-4,5-bis(dibenzylphospho)-*myo*-inositol] Phosphate (5). The crude benzyl *N*-Cbz-3-amino-1-propyl 1-(2,3,6-tri-*O*-benzyl-*myo*-inositol) phosphate (430 mg, 0.53 mmol) was dried up evaporating in vacuo with 1 mL of DMF. The residue was dissolved in 8 mL of dry methylene chloride, and dibenzyloxy(*N,N*-diisopropylamino)phosphine[9,15] (480 mg, 1.4 mmol) in 2 mL of methylene chloride and tetrazole (175 mg, 2.5 mmol) was added. The mixture was stirred at room temperature for 3 h, then cooled to −40 °C (dry ice–acetonitrile bath), and treated with a suspension of *m*-chloroperbenzoic acid (0.5 g) in 4 mL of methylene chloride. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 30 min. The solution was diluted with 75 mL of methylene chloride, washed (2 × 10 mL of 10% Na_2SO_3, 2 × 10 mL of saturated $NaHCO_3$, 10 mL of water), dried ($MgSO_4$), filtered, and concentrated to give 1.0 g of a colorless oil. The crude product was chromatographed on silica gel by using ethyl acetate–hexane (3:1) to give 600 mg of purified product **5** in 83% yield: the ^{31}P NMR ($CDCl_3$) showed peaks at δ −2.4, −2.77, and −2.81 ppm in a 4:1:1 ratio (mixture of diastereomers); ^1H NMR ($CDCl_3$) δ 7.0–7.4 (45 H, m), 5.00 (2 H, s), 4.0–5.0 (21 H, m), 3.75 (2 H, m), 3.44 (1 H, m), 2.92 (2 H, m), 1.45 (2 H, m). MS (FAB) *m/q* 1332 (M + 1).

1-*O*-(3-Aminopropyl-1-phospho)-*myo*-inositol 4,5-(bis)phosphate (1a). Benzyl *N*-Cbz-3-amino-1-propyl 1-(2,3,6-tri-O-benzyl-4,5-bis(dibenzylphospho)-*myo*-inosityl) phosphate (**5**; 320 mg, 0.22 mmol) was dissolved in 100 mL of 95% ethanol, and 200 mg of 10% Pd–C catalyst was added. Hydrogenolysis at an initial hydrogen pressure of 3 atm was allowed to proceed at room temperature for 6.5 h. The catalyst was removed by filtration through Celite and washed with 30 mL of 2:1 ethanol–water followed by 15 mL of water. The combined filtrates were brought to pH 8.0 with a few drops of concentrated ammonium hydroxide and the solvent was removed in vacuo. The residue was dissolved in 3 mL of water and applied to a 6 × 1.4 cm column of Bio-Rad Chelex 100 resin (Na form). The column was eluted with 25 mL of water. Evaporation afforded 130 mg (75%) of the sodium salt of **1a** as a colorless glass: the ^1H-coupled ^{31}P NMR (D_2O) consisted of three peaks at δ 5.58 (d, *J* = 7.8 Hz), 5.51 (d, *J* = 7.8 Hz), and 0.49 ppm (m) in a 1:1:1 ratio; ^1H NMR (D_2O) δ 3.7–4.15 (7 H, m), 3.6 (1 H, dd), 3.02 (2 H, t), 1.89 (2 H, m); ^{13}C NMR (D_2O) δ 76.7 (1 C, s), 76.1 (1 C, d, *J* = 4.8 Hz), 74.7 (1 C, s), 71.6 (2 C, b), 70.5 (1 C, s), 63.5 (1 C, d, *J* = 5.1 Hz), 37.0 (1 C, s), 28.0 (12 C, s).

1-*O*-[*N*-(4-Azidosalicyloxy)-3-aminopropyl-1-phospho]-*myo*-inositol 4,5-(Bisphosphate (1b). 1-*O*-(3-Aminopropyl-1-phospho)-*myo*-inositol 4,5-biosphosphate (**1a**; 11.4 mg, 20 μmol) was dissolved in 500 μL of 0.25 M triethylammonium bicarbonate (TEAB) buffer and (under a red safe light) *N*-hydroxysuccinimido 4-azidosalicyclic acid (13.6 mg, 50 μmol) dissolved in 250 μL of DMF was added. An initially clear solution resulted, but after a few minutes a solid precipitated. The mixture was stirred in the dark for 4.5 h. Almost everything was in solution at this point. The solvents were evaporated in vacuo and the residue was evaporated once with 2 mL of water. The residue was dissolved in 3 mL of water and applied to a 10 × 2.2 cm column of DEAE cellulose (HCO_3^- form). The column was washed with 20 mL of water and then eluted with 10 mL of 0.2 M TEAB, 10 mL of 0.3 M TEAB, 2 × 10 mL of 0.4 M TEAB, and 2 × 10 mL of 0.6 M TEAB. The product eluted in the 0.4 and 0.6 M buffer. Analysis of these buffer fractions by ion-exchange HPLC (25-cm Partisil 10 SAX, 0.5 M KH_2PO_4 at pH 4.5, 1 mL/min, UV detection at 270 nm) showed a single product with a retention time of 12 min. The buffer fractions containing the product were evaporated in vacuo and residual buffer was removed by evaporating with several small volumes of methanol to give 13.3 mg of photolabel **1b** as a colorless glass: ^1H NMR (D_2O) δ 7.62 (1 H, d), 6.60 (1 H, d), 6.53 (1 H, s), 2.9–4.1 (10 H, m), 1.80 (2 H, m). ^{31}P NMR (D_2O) δ 0.52, 4.45 (1:2 ratio).

Preparation of the Affinity Matrix (1c) by Treatment of Affi-Gel 10 with 1-*O*-(3-Aminopropyl-1-phospho)-*myo*-inositol 4,5-Bisphosphate. Affi-Gel 10 resin (10-mL slurry; Bio-Rad) was transferred to a sintered glass funnel and the 2-propanol storage solvent removed by gentle suction. The *N*-hydroxysuccinimide activated ester resin was washed with 25 mL of ice water in small portions, taking care to keep it from drying out. It was then added to an ice-cold solution of 1-*O*-(3-aminopropyl-1-phospho)-*myo*-inosito 4,5-bisphosphate (17 mg) and sodium bicarbonate

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(40 mg) in 4 mL of water. The mixture was stirred at 0 °C for 24 h. The modified resin (**1a**) was isolated by filtration on a sintered glass funnel and washed with 5 × 20 mL of ice water. It was stored at 4 °C as a suspension in water.

General Procedures (Biochemical). Protein was determined by the Bradford method.[16] SDS-PAGE was performed by the method of Laemmli,[17] and the gels were stained with silver.[18]

Competition of Compounds with [^{3}H]Ins(1,4,5)P_3 Binding. Ins(1,4,5)P_3 binding to washed rat cerebellar membranes was measured as described.[12b] Tissue from freshly sacrificed Sprague–Dawley rats (200–300 g) was homogenized (50 mg wet weight/mL of buffer) with a Brinkmann Polytron (setting 6; 10 s) at 4 °C in buffer A containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, 100 μg/mL phenylmethanesulfonyl fluoride, 10 μg/mL *N*-*p*-tosylarginine methyl ester, and 5 μg/mL each of leupeptin and aprotinin. Homogenates were centrifuged (15 min, 30000*g*), the supernatants were discarded, and the pellets were resuspended in the same volume of buffer. The sequence of homogenization, centrifugation, and resuspension of the membrane pellets was repeated twice to provide the washed membrane fraction.

Binding reactions consisted of 50 μL of washed membranes, 5 nM [^{3}H]Ins(1,4,5)P_3 (20 Ci/mmol), 50 μL of test compound at each concentration and buffer A to a volume of 0.5 mL. Reactions were allowed to reach equilibrium at 4 °C for 10 min and terminated by centrifugation (5 min, 12000*g*). Unbound ligand in the supernatant was removed by aspiration and bound radioactivity in the pellets was measured by liquid scintillation chromatography in 5 mL of formula 963 (New England Nuclear) following membrane pellet solubilization with 100 μL of 1 N NaOH. Specific binding represented less than 10% of added radioactivity. Nonspecific binding was determined in the presence of 5 μM unlabeled Ins(1,4,5)P_3. K_i values were determined by using the EBDA binding data analysis program.

[^{3}H]Ins(1,4,5)P_3 Binding to the Solubilized Receptor. Ins(1,4,5)P_3 binding to detergent-solubilized fractions and to purified Ins(1,4,5)P_3 receptor was determined by a poly(ethylene glycol) (6000 PEG) precipitation assay as follows: Samples were incubated to equilibrium (10 min) at 4 °C with 5 nM [^{3}H]Ins(1,4,5)P_3 in a reaction volume of 0.5 mL. Protein containing bound Ins(1,4,5)P_3 was precipitated with 50 μL of 0.5% γ-globulin and 0.75 mL of ice-cold 30% PEG. After an additional 5 min, the samples were centrifuged (10 min, 12000*g*), supernatants were removed, the pellets were solubilized with 100 μL of 1 N NaOH, and radioactivity was measured in 5 mL of formula 693. Nonspecific binding was determined in the presence of 5 μM unlabeled Ins(1,4,5)P_3.

Evaluation of Compounds in Mediating Calcium Flux in Ins(1,4,5)P_3 Receptor Reconstituted Lipid Vesicles. Ins(1,4,5)P_3 receptor reconstituted lipid vesicles were prepared as described,[13] and the compounds were evaluated for their ability to induce calcium flux. Calcium flux was measured by incubating 85 μL of reconstituted vesicles with 2 μCi of $^{45}Ca^{2+}$, in the presence or absence of Ins(1,4,5)P_3 or the compound tested, in a final incubation volume of 100 μL for 10 s. The flux assay was stopped by the addition of a 3-fold excess of buffer containing 50 mM NaCl, 50 mM KCl, 20 mM Tris-HCl (pH 7.4, 25 °C) 0.3 mM $CaCl_2$, 5 mM $MgSO_4$, 1 mM $CdCl_2$, and 100 μg/mL heparin (Sigma). The vesicles were then immediately loaded on to a 3-mL column of Tris-Dowex (Sigma, Dowex 50-W X-8, pH 8.0) preequilibrated with buffer containing 200 mM sucrose, 20 mM Tris-HCl (pH 7.4, 25 °C), and 0.3% bovine serum albumin (BSA). The column was washed with 3.5 mL of the same buffer (without BSA) to collect the vesicles. Intravesicular $^{45}Ca^{2+}$ content was determined by liquid scintillation spectrometry. EC_{50} values were calculated from the drug concentration required to release a half-maximal amount of $^{45}Ca^{2+}$ from the reconstituted vesicles.

Evaluation of Compounds as Substrates for Ins(1,4,5)P_3 5-Phosphatase or 3-Kinase. The synthesized compounds were evaluated as potential substrates or inhibitors of rat cerebellar Ins(1,4,5)P_3 5-phosphatase and 3-kinase activities as described.[14]

Evaluation of the Aminopropyl Ins(1,4,5)P_3 Affinity Resin in Purification of Ins(1,4,5)P_3 Receptor. The use of the aminopropyl Ins(1,4,5)P_3 affinity resin was compared to standard purification procedures previously published for purifying Ins(1,4,5)P_3 receptors from rat cerebellum.[2c] Five freshly dissected rat cerebella (1.5 g) were homogenized in 25 mL of buffer A and washed membranes were prepared as described above. All procedures were conducted at 4 °C. Washed cerebellar membranes were solubilized with Triton X-100 detergent (final concentration 1%) with gentle stirring for 30 min followed by centrifugation (45 min, 45000*g*). The supernatant was collected, adjusted to 0.25 M NaCl, and passed over a heparin–agarose column (1-mL packed volume) that had been equilibrated with buffer A containing 0.25 M NaCl and 0.1% Triton X-100. The column was washed twice with five column volumes of buffer A containing 0.25 M NaCl and 0.1% Triton X-100, followed by elution with four column volumes of buffer A containing 0.5 M NaCl and 0.1% Triton X-100. The heparin–agarose column eluate was divided into two portions. Half was applied to the concanavalin A–Sepharose resin and half was applied to the aminopropyl-Ins(1,4,5)P_3 affinity resin **1c**.

Eluate from the heparin column was adjusted to 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubated with 0.5 mL of packed concanavalin A–Sepharose for 1 h on a rotator. After 1 h, the resin was poured into a column, washed twice with five column volumes of buffer A (containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% Triton X-100) and eluted batchwise for 2 h with 2 mL of buffer A containing 2 mM EDTA, 1 M methyl α-mannoside, and 0.1% Triton X-100.

The other half of the heparin eluate was diluted to a final concentration of 0.25 M NaCl and was incubated batchwise with 2 mL of aminopropyl Ins(1,4,5)P_3 affinity resin for 20 min on a rotator. The column was washed three times with four column volumes of buffer A containing 0.25 M NaCl and 0.1% Triton X-100 and eluted batchwise for 30 min with 2 mL of either buffer A containing 0.6 M NaCl or 10 mM phytic acid (Ins(1,2,3,4,5,6)P_6). Both elution conditions provided similar results.

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