Synthesis of Affinity Ligands and Radioactive Probes for Isolation and Study of *myo*-Inositol 1,4,5-Trisphosphate Binding Proteins†

Arvind N. Jina,‡,§ John Ralph,‖,⊥ and Clinton E. Ballou*,‡
Departments of Biochemistry and Chemistry, University of California, Berkeley, California 94720
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ABSTRACT: To synthesize an affinity matrix for isolation of D-*myo*-inositol 1,4,5-trisphosphate binding proteins, racemic 3-cyclohexene-1-carboxaldehyde was oxidized and converted to a mixture of *trans*-3,4-dihydroxycyclohexane-1-carboxylic acid methyl ester isomers, which was phosphorylated and separated into (±)-(1*R*,3*R*,4*R*)- and (±)-(1*R*,3*S*,4*S*)-*trans*-3,4-bis[(diphenoxyphosphoryl)oxy]cyclohexane-1-carboxylic acid methyl esters. Each of these racemic compounds was hydrogenolyzed and reacted with ethylenediamine to give a monoamide, *N*-(2-aminoethyl)-bis(phosphonyloxy)cyclohexane-1-carboxamide, that was coupled to cyanogen bromide activated Sepharose 4B to provide the desired affinity matrices. The intermediate *trans*-3,4-bis[(diphenoxyphosphoryl)oxy]cyclohexane-1-carboxylic acid methyl ester was also reduced with lithium borotritide to give the (hydroxy[^{3}H]methyl)cyclohexane derivative, which was phosphorylated and hydrogenolyzed to yield *trans*-3,4-bis(phosphonyloxy)-1-[(phosphonyloxy)[^{3}H]methyl]cyclohexane, a radiolabeled analogue of inositol 1,4,5-trisphosphate. The carboxamide was also coupled to 4-azidosalicylic acid, and the product was iodinated to provide a ^{125}I-radiolabeled photoactivatable cross-linking derivative of cyclohexanediol bisphosphate.

The role of D-*myo*-inositol 1,4,5-trisphosphate (**1**) (IP_3;[1] Figure 1), as a second messenger for calcium mobilization from intracellular stores, in particular, the endoplasmic reticulum, is now generally accepted (Streb et al., 1983; Berridge & Irvine, 1984). IP_3 probably stimulates the release of calcium by acting through a specific receptor on the endoplasmic reticulum membrane that is related to, or is an integral part of, the calcium channel.

It has been found (Burgess et al., 1984; Irvine et al., 1984) that, of the inositol phosphates tested, only those bearing phosphate groups on both the 4- and 5-positions of the ring are capable of stimulating the release of calcium. The approximate order of potency was 1,4,5-IP_3 > 2,4,5-IP_3 > 4,5-IP_2, which suggested that the trans-vicinal phosphates on positions 4 and 5 are essential for the calcium mobilizing effect of IP_3 and that a localized negative charge in the form of a phosphate group at position 1 enhances this activity. Preliminary studies[2] showing that *trans*-cyclohexane-1,2-diol bisphosphate (**2**) (Brown & Usher, 1965), a structural analogue of 4,5-IP_2, can compete with IP_3 binding to endoplasmic reticulum membranes are in agreement with the above observations. On the basis of this evidence, we concluded that the related analogue **3** would be suited for synthesis of derivatives to be used in isolation of the receptor by affinity chromatography, and this report describes our efforts in that direction. A preliminary report has appeared (Jina et al., 1989), and studies of a related nature have been published (Henne et al., 1988; Polokoff et al., 1988; Ishimatsu et al., 1988).

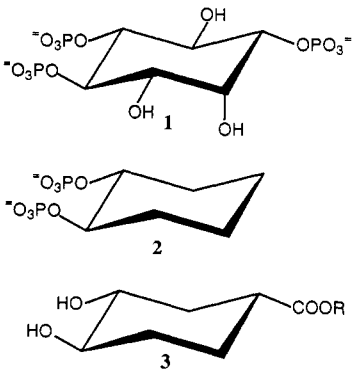

FIGURE 1: Structures of D-*myo*-inositol 1,4,5-trisphosphate and related cyclohexanediol analogues.

Experimental Procedures

General Methods. All NMR spectra were recorded on a Bruker AM-500 spectrometer, with an ASPECT 3000 computer, using a 5-mm broad-band multinuclear probe head. Acquisition and digitization parameters varied according to the nature of the experiment. Proton and carbon spectra were run in deuteriochloroform ($DCCl_3$) with tetramethylsilane (TMS, δ 0.0) as internal standard, or in D_2O using dioxane (δ 3.7) as the internal reference. Data for ^{1}H NMR will be presented as follows: δ (number of protons, multiplicity, coupling constants, assignment). Ultraviolet spectra (UV) were obtained on a Carl Zeiss Model PMQ II spectrophotometer and infrared spectra on a Perkin-Elmer Model 710B spectrometer in chloroform solutions unless otherwise noted.

Thin-layer chromatography (TLC) was done on precoated silica gel 60 F-254 plates (0.25 mm, E. Merck). Chromatograms were viewed under UV light or were developed by spraying with chromic acid followed by heating. Descending paper chromatography was carried out on Whatman No. 1 paper. Phosphorus-containing compounds were detected with the reagent of Bandurski and Axelrod (1951) by exposing the sprayed chromatograms to UV light for 0.5 h. Column

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* Address correspondence to this author.
‡ Department of Biochemistry.
§ Present address: Stanford Research Institute, Menlo Park, CA 94205.
‖ Department of Chemistry.
⊥ Present address: Dairy Forage Research Center, USDA, University of Wisconsin—Madison, Madison, WI 53706.

[1] Abbreviations: IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_2, *myo*-inositol 4,5-bisphosphate; Ph, phenyl; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.
[2] G. Denis and C. E. Ballou, unpublished results.

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chromatography was performed on silica gel (silica gel 60, 70–230 mesh ASTM, E. Merck). Preparative high-performance liquid chromatography (HPLC) was done on columns of LiChroprep RP-18 (25–40 mm, E. Merck) while reactions were monitored by HPLC on an analytical Supelco LC-18 column. Gel filtration was done on a Bio-Gel P-2 (200–400 mesh) column and anion-exchange chromatography on QAE-Sephadex A-25. Phosphorus-containing fractions were detected by using the Ames (1966) method. Liquid reagent mixtures are expressed throughout an a v/v basis. Elemental analyses were done in the Department of Chemistry, University of California, Berkeley. Radioactive analogues were characterized by comparing their chromatographic properties with those of the unlabeled compounds, whereas NMR data and combustion analyses are reported for the latter.

trans-Cyclohexane-1,2-diol Bisphosphate (**2**). To 2.5 g (21.5 mmol) of *trans*-cyclohexane-1,2-diol in 10 mL of dry pyridine at 0 °C was added dropwise a solution of diphenyl chlorophosphate (14.5 g, 54.0 mmol) in 10 mL of pyridine. The reaction mixture was stirred for 0.5 h at 0 °C after which it was left stirring for 24 h at 25 °C. Water (2 mL) was added, the mixture was stirred for an additional 1 h, and then it was evaporated to dryness under vacuum at 25 °C. The residue was dissolved in 150 mL of chloroform, and the solution was washed successively with water (100 mL), dilute hydrochloric acid (100 mL), saturated sodium hydrogen carbonate solution (50 mL), and water (100 mL) after which it was dried over anhydrous sodium sulfate. The solution was decolorized with activated charcoal, filtered through Celite, and concentrated to a clear syrup under reduced pressure (86%). The syrupy bis[(diphenoxyphosphoryl)oxy]cyclohexanediol (5.4 g) was dissolved in absolute ethanol (100 mL) and hydrogenated at room temperature and 1 atm over platinum oxide (1.35 g) until uptake of hydrogen ceased at 95–100% of the theoretical value. The solution was decanted from the catalyst and filtered through a pad of Celite, and the pH was adjusted to about 9.5 with redistilled cyclohexylamine. Filtration of the precipitate followed by crystallization from ethanol furnished the pure tricyclohexylammonium salt of the bisphosphate (75%): mp 216–224 °C. Anal. Calcd for $C_{24}H_{53}N_3O_8P_2$: C, 50.3; H, 9.3; N, 7.3; P, 10.6. Found: C, 50.2; H, 9.2; N, 7.1; P, 10.8.

(±)-(*1R,3R,4R*)-*trans-3,4-Dihydroxycyclohexane-1-carboxylic Acid Methyl Ester* (**7a**) *and* (±)-(*1R,3S,4S*)-*trans-3,4-Dihydroxycyclohexane-1-carboxylic Acid Methyl Ester* (**7b**). To a stirred suspension of 70% hydrogen peroxide (1.66 mL, 44.0 mmol) in 20 mL of methylene chloride in an ice bath was added trifluoroacetic anhydride (7.44 mL, 52.8 mmol). The resulting solution was stirred for 15 min in the cold, then carefully transferred to a dropping funnel, and added dropwise over a 15-min interval to a solution of 3-cyclohexenecarboxaldehyde (**4**; 2.3 mL, 20 mmol) and triethylammonium trifluoroacetate (1.98 mL, 10 mmol) in 20 mL of methylene chloride at 0 °C. The reaction mixture was stirred at 25 °C for 24 h and then concentrated to dryness under vacuum. The residue was dissolved in methanol–water (1:1 v/v), and the solution was treated with a slight excess of Dowex 50 (H^+). The resin was removed by filtration and washed with methanol–water (2 × 100 mL), and the filtrate was concentrated under reduced pressure to give a crude oil free of triethylammonium trifluoroacetate. Methanolysis and esterification were carried out by dissolving the oil in dry methanol (60 mL), adding trifluoroacetic acid (2 mL), stirring the solution for 24 h at room temperature, and evaporating it to dryness under vacuum. The inseparable diastereomeric diols **7a** and **7b** were obtained as a clear oil (55%), ν_{max} 3420 (OH), 1720 (ester) cm^{-1}, following chromatography on silica gel (ethyl acetate) or preparative HPLC (7.5% methanol). Anal. Calcd for $C_8H_{14}O_4$: C, 55.2; H, 8.0. Found: C, 55.3; H, 8.1.

(±)-(*1R,3R,4R*)-*trans-3,4-Bis*[(*diphenoxyphosphoryl*)*oxy*]*cyclohexane-1-carboxylic Acid Methyl Ester* (**9a**) *and* (±)-(*1R,3S,4S*)-*trans-3,4-Bis*[(*diphenoxyphosphoryl*)*oxy*]-*cyclohexane-1-carboxylic Acid Methyl Ester* (**9b**). To the diol mixture (**7a** and **7b**; 2.2 g, 12.6 mmol) in 10 mL of dry pyridine at 0 °C was added dropwise a solution of diphenyl chlorophosphate (10.1 g, 37.5 mmol) in 10 mL of pyridine. The mixture was stirred at 0 °C for 0.5 h, allowed to warm to room temperature, and stirred for 24 h. The reaction was quenched by adding 2 mL of water and stirred for an additional 1 h, after which it was evaporated to dryness under vacuum. The product was dissolved in chloroform (150 mL), the solution was washed successively with water (100 mL), dilute hydrochloric acid (100 mL), saturated sodium carbonate solution (50 mL), and water (100 mL), and the solution was dried over anhydrous sodium sulfate. The solution was decolorized with activated charcoal, filtered through Celite, and concentrated to a clear syrup under vacuum (95%). Chromatography on either silica gel (ethyl acetate–hexane, 1.5:2) or HPLC (77% methanol) furnished the pure diphosphorylated diastereomers **9a** and **9b** in a 3:2 ratio, respectively. The major and more polar diequatorial isomer (**9a**), which crystallized on standing, was characterized as follows: mp 79–81 °C; ^1H NMR δ 7.20 (20 H, m, aromatic H), 4.65 (1 H, m, H-3), 4.62 (1 H, m, H-4), 3.65 (3 H, s, OCH_3), 2.55 (1 H, bd, $J_{2e,2a}$ = 12 Hz, H-2e), 2.45 (1 H, m, H-1), 2.40 (1 H, m, H-5e), 2.00 (1 H, bd, $J_{6e,6a}$ = 12 Hz, H-6e), 1.77 (1 H, dt, $J_{2a,2e}$ 12 Hz; $J_{2a,3} = J_{2a,1}$ = 10 Hz, H-2a), 1.58 (1 H, m, H-5a), 1.55 (1 H, m, H-6a); ^{13}C NMR (125.8 MHz) δ 173.5 (s, C=O), 150.5 (s, C_1-aromatic), 129.7 (d, C_3- and C_5-aromatic), 125.4 (d, C_4-aromatic), 120.1 (d, C_2- and C_6-aromatic), 79.4 (d, C_3), 78.9 (d, C_4), 52.0 (q, OCH_3), 40.2 (d, C_1), 33.5 (t, C_2), 30.0 (t, C_5), 25.8 (t, C_6). Anal. Calcd for $C_{32}H_{32}O_{10}P_2$: C, 60.19; H, 5.02; P, 9.72. Found: C, 59.92; H, 5.07; P, 9.59. The less polar minor diaxial isomer (**9b**) gave the following data: ^1H NMR δ 7.24 (20 H, m, aromatic H), 4.87 (1 H, m, H-3), 4.76 (1 H, m, H-4), 3.66 (3 H, s, OCH_3), 2.62 (1 H, m, H-1), 2.05 (2 H, m, H-2e, H-2a), 1.88 (2 H, m, H-5e, H-5a), 1.76 (2 H, m, H-6e, H-6a); ^{13}C NMR (125.8 MHz) δ 174.6 (s, C=O), 150.3 (s, C_1-aromatic), 129.8 (d, C_3- and C_5-aromatic), 125.4 (d, C_4-aromatic), 120.0 (d, C_2- and C_6- aromatic), 74.7 (d, C_3), 74.4 (d, C_4), 51.8 (q, OCH_3), 36.4 (d, C_1), 28.9 (t, C_2), 25.8 (t, C_5), 22.1 (t, C_6). Anal. Calcd for $C_{32}H_{32}O_{10}P_2$: C, 60.19; H, 5.02; P, 9.72. Found: C, 59.60; H, 5.02; P, 10.07.

(±)-(*1R,3R,4R*)-*trans-N-*(*2-Aminoethyl*)*-3,4-bis*(*phosphonyloxy*)*cyclohexane-1-carboxamide* (**11a**). The major phosphorylated isomer (**9a**; 1.5 g) was hydrogenated in absolute ethanol at 25 °C and 1 atm over platinum oxide (0.5 g) for 24 h. The reduction was monitored by HPLC and stopped when the peak for starting material was no longer discernible and an NMR spectrum of the crude product indicated the absence of aromatic signals. The solution was decanted from the catalyst and filtered through a pad of Celite, and the pH was adjusted to 9.5 with redistilled ethylenediamine. Evaporation to dryness at 40 °C under reduced pressure gave the ethylenediammonium salt of the diphosphate (**10a**) in quantitative yield, which was used immediately without purification in the following condensation reaction. To 1.2 g of the of the crude diphosphate (**10a**) was added 18 mL of redistilled ethylenediamine, 3 mL of methanol, and 3 mL of water to dissolve the salt completely. The reaction

was stirred in a sealed flask for 5 days at room temperature and then concentrated to dryness under vacuum (0.1 mmHg) at 30 °C to give 1.38 g of a yellowish solid. The crude product was desalted on a Bio-Gel P-2 column, and the major fraction was pooled, evaporated to dryness, and applied at pH 6.5 to a QAE-Sephadex A-25 column that was eluted by using a 0.6 M linear lithium chloride gradient in 2 mM Tris-HCl buffer (pH 7). The major fraction was pooled, concentrated under vacuum, and desalted on a Bio-Gel P-2 column. Evaporation of the eluate afforded the desired monoamide (**11a**) as its amine hydrochloride: ^{1}H NMR (D_2O) δ 3.68 (2 H, m, H-3, H-4), 3.20 (2 H, m, $CH_{2\beta}NH_3$·Cl), 2.85 (2 H, t, $CONHCH_{2\alpha}$), 2.15 (1 H, tt, $J_{1,2e} = J_{1,6e} = 4$ Hz, $J_{1,2a} = J_{1,6a} = 11$ Hz, H-1), 1.95 (1 H, m, H-2e), 1.88 (1 H, m, H-5e), 1.56 (1 H, m, H-6e), 1.28 (1 H, m, H-2a), 1.20 (2 H, m, H-6a, H-5a); ^{13}C NMR (125.8 MHz, D_2O) δ 178.4 (s, C=O), 76.1 (d, C_4), 42.0 (d, C_1), 38.8 (t, C_β), 36.7 (t, C_α), 34.6 (t, C_2), 30.7 (t, C_5), 26.4 (t, C_6); ^{31}P NMR (202.5 MHz, D_2O) δ 4.69 (C_3-P), 4.58 (C_4-P). Anal. Calcd for $C_9H_{19}N_2O_9P_2ClLi_2 \cdot 2H_2O$: C, 24.21; H, 5.15; N, 6.27; P, 13.90; Li, 3.11. Found: C, 24.50; H, 4.97; N, 6.04; P, 14.40; Li, 3.60.

(±)-(*1R,3S,4S*)-*trans*-*N*-(*2-Aminoethyl*)-*3,4-bis*(*phosphonyloxy*)*cyclohexane-1-carboxamide* (**11b**). The corresponding minor phosphorylated product (**9b**; 1.0 g) was hydrogenated, condensed with ethylenediamine, and purified as described above for compound **11a**, to give the crystalline diastereomeric monoamide **11b** as its amine hydrochloride: ^{1}H NMR (D_2O) δ 4.19 (1 H, m, H-3), 4.06 (1 H, m, H-4), 3.44 (2 H, m, $CH_{2\beta}NH_3$·Cl), 3.07 (2 H, t, $CONHCH_{2\alpha}$), 2.63 (1 H, tt, $J_{1,2e} = J_{1,6e} = 4$ Hz, $J_{1,2a} = J_{1,6a} = 11$ Hz, H-1), 1.82 (4 H, m, H-2a, H-2e, H-5a, H-5e), 1.68 (1 H, m, H-6e), 1.50 (1 H, m, H-6a). ^{13}C NMR (125.8 MHz, D_2O) δ 182.10 (s, C=O), 71.4 (d, C_3), 70.7 (d, C_4), 40.2 (t, C_β), 39.4 (d, C_1), 38.12 (t, C_α), 30.6 (t, C_2), 26.6 (t, C_5), 23.3 (t, C_6); ^{31}P NMR (202.5 MHz, D_2O) δ 4.14 (C_3-P), 4.00 (C_4-P). Anal. Calcd for $C_9H_{19}N_2O_9P_2ClLi_2 \cdot 3H_2O$: C, 23.27; H, 5.38; N, 6.03; P, 13.36. Found: C, 23.11; H, 5.13; N, 5.47; P, 13.19.

Coupling of **11a** *and* **11b** *to CNBr-Activated Sepharose 4B.* Freeze-dried CNBr-activated Sepharose (0.5 g, Sigma) was swelled in cold 1 mM hydrochloric acid (5 mL) for 20 min, collected on a sintered glass filter, and washed with 150 mL (5 × 30 mL) of the same solution. The swollen gel (1 mL) was immediately transferred to a 1.5-mL solution of the ligand (**11a**, 8.5 mg) in 0.1 M sodium hydrogen carbonate (pH 8.3) containing 0.5 M sodium chloride, and the tube was mixed gently end over end for 2 h at room temperature. The mixture was filtered and washed with 30 mL of the coupling buffer, and the remaining active groups were blocked by suspending the gel in 3 mL of 1 M ethanolamine (pH 8) for 2 h at 25 °C. The gel was collected on a filter and washed successively with 30 mL of coupling buffer and 30 mL of 0.1 M acetate buffer, pH 4.0, containing 0.5 M sodium chloride, and again with 30 mL of coupling buffer. The wash cycle was repeated three times, and the resulting ligand–Sepharose conjugate was stored in saline solution at 4 °C. A coupling efficiency of 60% was estimated on the basis of a phosphate assay.

Coupling of **11a** *to Epoxy-Activated Sepharose 6B.* Freeze-dried epoxy-activated Sepharose 6B (0.5 g, Sigma) was swelled in distilled water for 30 min, collected on a sintered glass filter, and washed extensively with distilled water (800 mL) and then with 50 mL of coupling solution (0.1 M sodium carbonate, pH 11.2). The swollen gel (2 mL) was transferred to a 2.0-mL solution of the ligand (**11a**; 9.2 mg) in 0.1 M sodium carbonate (pH 11.2), and the sealed tube was mixed gently end over end for 24 h at 37 °C. The reacted gel mixture was collected on a sintered glass filter and washed with 60 mL of the coupling buffer, followed by 100 mL of water, 60 mL 0.1 M sodium hydrogen carbonate, pH 8.0, containing 0.5 M sodium chloride, and 60 mL of 0.1 M sodium acetate, pH 4.0, containing 0.5 M sodium chloride. The gel was resuspended in 2 mL of the coupling buffer, and the remaining active groups were blocked by adding 3 mL of 1.0 M ethanolamine and mixing the gel end over end overnight at 37 °C. The gel was collected on a sintered glass filter and washed successively with 50 mL of coupling buffer, 40 mL of 0.1 M sodium hydrogen carbonate, pH 8.0, containing 0.5 M sodium chloride, 40 mL of 0.1 M sodium acetate, pH 4.0, containing 0.5 M sodium chloride, 50 mL of water, and 30 mL of 0.9% sodium chloride solution. The resulting ligand–Sepharose conjugate was stored in saline solution, and a coupling efficiency of 52% was estimated on the basis of a phosphate assay.

(±)-(*1R,3R,4R*)-*trans*-*3,4-Bis*[(*diphenoxyphosphoryl*)*oxy*]-*1*-(*hydroxymethyl*)*cyclohexane* (**13a**). To 75.9 mg. [0.12 mmol] of (±)-(1*R*,3*R*,4*R*)-*trans*-3,4-bis[(diphenoxyphosphoryl)oxy]cyclohexane-1-carboxylic acid methyl ester (**9a**) in 3 mL of absolute ethanol were added 2.32 mg (0.061 mmol, 25 mCi) of sodium borotritide (NEN, specific activity 407.0 mCi/mmol), 2.32 mg (0.061 mmol) of sodium borohydride, and 10.3 mg (0.12 mmol) of anhydrous lithium bromide. The reaction was stirred for 72 h at room temperature and monitored periodically by TLC on silica gel (ethyl acetate–hexane, 2:1). When the starting material had disappeared completely, the reaction was quenched with a few drops of water, stirred for a further 2 h, and evaporated to dryness under nitrogen. The product was isolated by chromatography on silica gel (ethyl acetate–hexane, 2:1), which yielded the alcohol as an oil. The reduced product was characterized by TLC and NMR. ^{1}H NMR δ 7.25 (20 H, m, aromatic H), 4.61 (2 H, m, H-3, H-4), 3.43 (2 H, m, CH_2OH).

(±)-(*1R,3R,4R*)-*trans*-*3,4-Bis*[(*diphenoxyphosphoryl*)*oxy*]-*1*-[[(*diphenoxyphosphoryl*)*oxy*]*methyl*]*cyclohexane* (**14a**). The alcohol **13a** was dissolved in 0.5 mL of dry pyridine, the solution was cooled to 0 °C in an ice bath, and excess diphenyl chlorophosphate (130.0 mg, 0.48 mmol) was added. the mixture was stirred at 0 °C for 30 min, allowed to warm up to room temperature, and stirred for 24 h. A thin-layer chromatogram of the reaction mixture indicated the quantitative conversion of the alcohol to the triphosphorylated derivative **14a**. The reaction was quenched by adding a few drops of water and stirred for an additional 1 h, after which it was evaporated to dryness under a stream of nitrogen. Chromatography of the crude product on a silica gel column (1.5 × 20 cm; ethyl acetate–hexane, 2:1) furnished the pure compound **14a** as a clear oil. ^{1}H NMR δ 7.22 (30 H, m, aromatic H), 4.61 (1 H, m, H-3), 4.52 (1 H, m, H-4), 4.04 (2 H, m, CH_2O), 2.35 (1 H, m, H-2e), 2.33 (1 H, m, H-5e), 1.84 (1 H, m, H-1), 1.73 (1 H, m, H-6e), 1.51 (1 H, m, H-5a), 1.31 (1 H, m, H-2a), 1.11 (1 H, m, H-6a). Anal. Calcd for $C_{43}H_{41}O_{12}P_3$: C, 61.28; H, 4.87; P, 11.04. Found: C, 60.98; H, 4.96; P, 10.61.

(±)-(*1R,3R,4R*)-*trans*-*3,4-Bis*(*phosphonyloxy*)-*1*-[(*phosphonyloxy*)*methyl*]*cyclohexane* (**15a**). The tris(diphenoxyphosphoryl) derivative **14a** was hydrogenated in absolute ethanol (10 mL) at 25 °C and 1 atm over platinum oxide (40 mg) for 24 h. The reduction was monitored by TLC and stopped after there was no discernible starting material present. The solution was decanted from the catalyst and filtered through a pad of Celite, which was washed with ethanol followed by a final wash with an ethanol–water mixture. The

pH of the filtrate was adjusted approximately to 9 with 0.1 M sodium hydroxide solution, and the solution was evaporated to dryness under nitrogen at 60 °C to give the sodium salt of the triphosphate, **15a**. The compound had a specific activity of 2.6 mCi/0.042 mmol of ligand (>62 mCi/mmol), which corresponded to a 10.4% incorporation. The overall yield of the triphosphate from the methyl ester **9a** was 35.3% on the basis of a phosphate determination. The labeled compound was characterized by chromatographic coelution with the nonradioactive derivative on a QAE-Sephadex A-25 column that was eluted with a 0.5 M linear lithium chloride gradient in 2 mM Tris-HCl buffer, pH 7. ^{1}H NMR δ 3.96 (1 H, m, H-3), 3.89 (1 H, m, H-4), 3.57 (2 H, m, CH_2O), 2.13 (2 H, m, H-2e, H-5e), 1.76 (1 H, m, H-6e), 1.70 (1 H, m, H-1), 1.39 (1 H, m, H-5a), 1.13 (1 H, m, H-2a), 1.00 (1 H, m, H-6a); ^{31}P NMR (202.5 MHz, D_2O) δ 6.77 (CH_2OP), 5.19 (C_3P), 5.11 (C_4P).

Coupling of **11a** *to Succinimido 4-Azidosalicylate.* To the diphosphate carboxamide **11a** (30 mg, 72 mmol) dissolved in 1.2 mL of 0.1 M $NaHCO_3$ was added succinimido 4-azidosalicylate (30 mg, 108 mmol) dissolved in 0.75 mL of acetonitrile. The reaction was left for 5 days at room temperature, when it was lyophilized and fractionated by ion exchange on a 15-mL QAE-Sephadex column (pH 6.5) using a 0.6 M linear LiCl gradient in 2 mM Tris-HCl buffer (pH 7). Two phosphate-containing fractions were obtained, a minor peak eluting at 0.2 M LiCl of unreacted **11a** and the major peak at 0.13 M LiCl corresponding to the coupled product. The latter fraction was lyophilized, desalted on a Bio-Gel P-2 column (2.0 × 100 cm), and evaporated to yield the desired product **16** as a solid: ^{1}H NMR δ 7.45 (1 H, d, H-6′, aromatic), 6.45 (1 H, d, H-5′, aromatic), 6.40 (1 H, s, H-3′, aromatic), 3.8 (2 H, m, H-3, H-4 cyclohexane ring), 3.3–3.2 (4 H, m, $CH_{2\alpha}$, $CH_{2\beta}$); UV (H_2O) λ_{max} = 306 nm.

Radioiodination of **16**. Iodination was done by a modified solid-phase procedure (Markwell, 1982). Iodobeads (66 beads, 36.4 μmol), washed with water (50 mL) and 0.1 M $NaHCO_3$ (2 × 50 mL) and then partially dried on Whatman No. 1 filter paper, were added to 10 mg (18.2 μmol) of **16** in 4 mL of 0.1 M $NaHCO_3$. $Na^{125}I$ (0.47 nmol, 1 mCi, 10 μL) was quickly added to the heterogeneous reaction mixture, which immediately changed from colorless to yellow. After 5 min of mixing on a Gyrotory shaker, the reaction was quenched with NaI (5.5 mg, 36.6 μmol) and left to stir for an additional 3 h. The reaction solution was transferred by pipet to a 50-mL round-bottom flask, the beads were washed with three 5-mL aliquots of 0.1 M $NaHCO_3$, and the combined solutions were lyophilized to give a solid residue.

The crude radioiodinated product was loaded on a Sephadex G-10 column (50 mL) equilibrated in 0.1 M NH_4HCO_3, and the column was eluted with 0.1 M NH_4HCO_3 (120 mL) and then with an ethanol–water mixture (1:1). The elution profile shows that most of the radioactivity and phosphate were eluted in a single peak by the ethanol–water mixture.

A parallel synthesis of the unlabeled analogue of **17** was undertaken for chromatographic comparison and spectral analysis. The labeled and unlabeled material were coeluted from a Sephadex G-10 column (not shown). The ^{1}H NMR spectrum of the unlabeled analogue showed a single singlet at δ 8.05 that integrated for one proton in the aromatic region of the spectrum indicative of diiodination. UV (H_2O) λ_{max} = 349 nm.

Results and Discussion

A retrosynthetic analysis of the proposed IP_3-related affinity ligand identifies three important steps: construction of a cyclohexanediol derivative with phosphate groups at positions 3 and 4, equivalent to the phosphate groups at positions 4 and 5 of D-*myo*-inositol 1,4,5-trisphosphate, that can be linked to an aliphatic spacer arm that can, in turn, be attached to a polymeric matrix. We decided that a dihydroxycyclohexane derivative, such as **3** (Figure 1), could serve as an appropriate building block, since it possesses the required trans-vicinal hydroxyl groups on positions 3 and 4 of the ring as well as an ester group at position 1 that can be extended into a spacer arm.

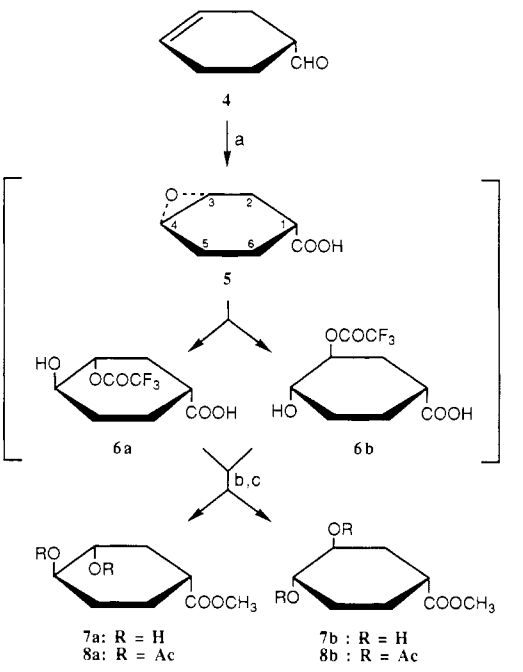

FIGURE 2: Scheme for synthesis of diols **7a** and **7b**. Reagents: (a) trifluoroperacetic acid–triethylammonium trifluoroacetate in dichloromethane, 25 °C, 2 h; (b) Dowex 50 (H^+) in methanol–water; (c) methanol–trifluoroacetic acid, 25 °C, 24 h.

The ester **3** is accessible from commercially available racemic 3-cyclohexene-1-carboxaldehyde (**4**, Figure 2). Following a modified procedure (Emmons et al., 1954; Emmons & Pagano, 1955), we transformed **4** stereospecifically, in a one-pot reaction, into the diastereomeric *trans*-diol esters **7a** and **7b** by oxidation with trifluoroperacetic acid. The intermediate epoxide **5**, without isolation, was cleaved by the trifluoroacetic acid to the diol monoesters **6a** and **6b**, with oxidation of the aldehyde group to the corresponding carboxylic acid occurring during the epoxidation step (Emmons & Lucas, 1955).

It has been reported (Emmons et al., 1954) that isolation of the intermediate hydroxytrifluoroacetates **6a** and **6b** by distillation is necessary to prevent contamination by triethylammonium trifluoroacetate, but this step led to a low yield of the diol in our hands. The reaction conditions were improved by treating the hydroxytrifluoroacetate residue with Dowex 50 (H^+) cation-exchange resin in a methanol-water mixture to remove the triethylamine. Filtration of the solution and evaporation of the solvents under reduced pressure to remove the trifluoroacetic acid afforded a product free of salt. Subsequent acid-catalyzed methanolysis and esterification furnished the diastereomeric diols **7a** and **7b**, which migrated together on TLC in various solvent systems. Conversion of the diols to their diacetates **8a** and **8b** also resulted in an inseparable mixture. The large coupling constant of about 30 Hz for H-1 in both isomers indicated that the ester group was in an equatorial orientation. The NMR spectrum of **8a** revealed it to be the diequatorial diacetate isomer, which pre-

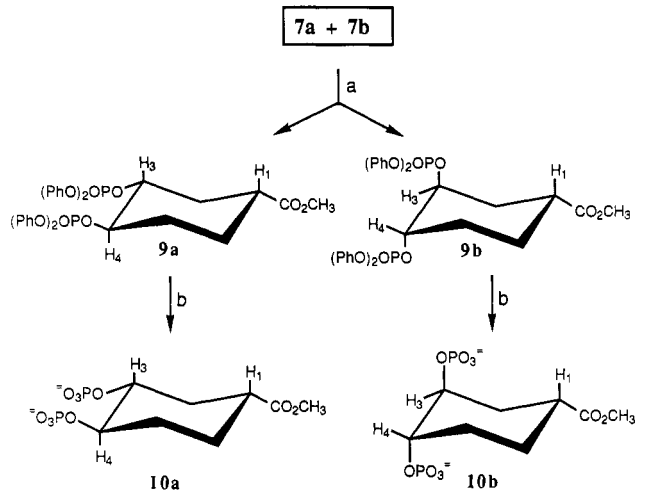

FIGURE 3: Scheme for synthesis of diphosphates **10a** and **10b**. Reagents: (a) diphenyl chlorophosphate in pyridine, 25 °C; (b) hydrogen–platinum oxide in ethanol, 25 °C.

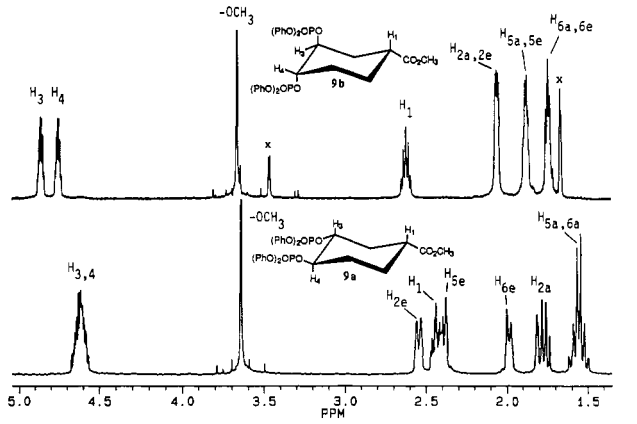

FIGURE 4: ^{1}H NMR spectra of **9a** and **9b** in deuteriochloroform. The characteristic splitting patterns of the ring protons distinguish between the diaxial (top) and diequatorial (bottom) isomers.

ponderated over the diaxial isomer, **8b**, in a 3:2 ratio. The diaxial H-3 and H-4 protons of compound **8a** produced a doublet of triplets at δ 4.84 with a combined coupling constant, J, of about 45 Hz, which is consistent for vicinal diaxial protons, whereas the diequatorial protons of the diacetate **8b** appeared as two multiplets at δ 5.02 for H-3 and δ 4.85 for H-4, each with a combined coupling constant of about 15 Hz.

Because the diequatorial orientation of the phosphate groups at positions 3 and 4 of the cyclohexane ring is a prerequisite for biological activity, it was necessary that we separate these isomers at some stage in the synthesis. Fortunately, after phosphorylation of the diol mixture **7a** and **7b** with diphenyl chlorophosphate in pyridine (Figure 3) we obtained compounds **9a** and **9b** that could be separated by chromatography and yielded the desired diequatorial phosphorylated compound as the major isomer. The H-3 and H-4 protons for this isomer appeared as a broad multiplet at δ 4.60, whereas isomer **9b** gave two distinct multiplets for H-3 at δ 4.90 and H-4 at δ 4.75 (Figure 4). Both isomers were characterized fully by NMR using ^{13}C, DEPT (Doddrell et al., 1982), COSY, and C–H correlated (Bax, 1983) spectroscopy. Catalytic hydrogenolysis of the phenyl groups of **9a** and **9b**, with platinum dioxide in ethanol or methanol, gave the racemic diphosphate compounds **10a** and **10b**. The NMR spectra and the HPLC characteristics of these crude diphosphates indicated that hydrogenolysis had gone to completion, and the products were used without further purification.

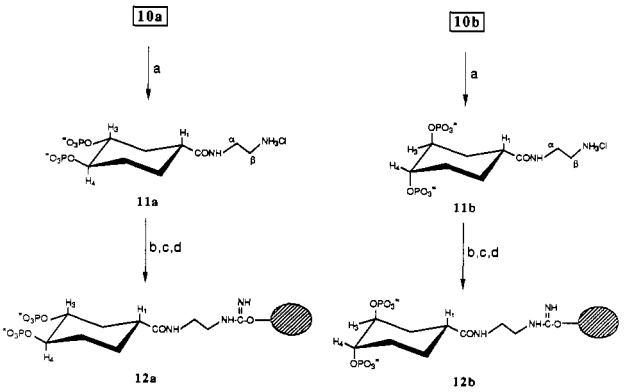

FIGURE 5: Scheme for synthesis of affinity matrices. Reagents: (a) ethylenediamine (80%) in methanol–water, 25 °C, 5 days; (b) cyanogen bromide activated Sepharose 4B; (c) 0.1 M sodium bicarbonate, pH 8.3, 25 °C, 2 h; (d) 1 M ethanolamine, pH 8.0, 25 °C, 2 h.

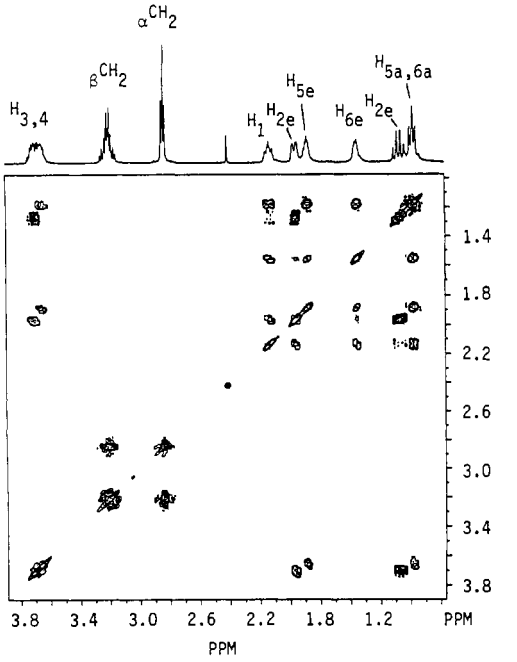

FIGURE 6: Two-dimensional COSY 45 proton spectrum of **11a**. See text for discussion of the cross-peaks.

The next phase of the synthesis involved attaching **10a** and **10b** to a flexible spacer arm that would also be suitable for coupling to an insoluble matrix. Condensation of the phosphate methyl esters **10a** and **10b** with 75% aqueous ethylenediamine (Hill & Aspinall, 1939; Aspinall, 1941) gave the monoamidated products **11a** and **11b** in good yield (Figure 5). The condensation required 4–5 days at room temperature, and attempts to accelerate the reaction by raising the temperature led to increased formation of the diamide (Leznoff, 1978). A more reactive substituent at C-1, such as an acyl chloride, enhanced formation of the unwanted diamide under various experimental conditions tried. For purification, the reaction mixture was concentrated under vacuum, desalted on a gel filtration column, and fractionated by anion-exchange chromatography with a linear gradient of lithium chloride. The major fractions obtained were desalted to furnish **11a** and **11b** as the hydrochlorides.

Compounds **11a** and **11b** were characterized fully by NMR spectroscopy. For example, the 500-MHz proton spectrum in D_2O of the diequatorial diphosphate isomer **11a** gave a single broad multiplet at δ 3.68 for the H-3 and H-4 ring

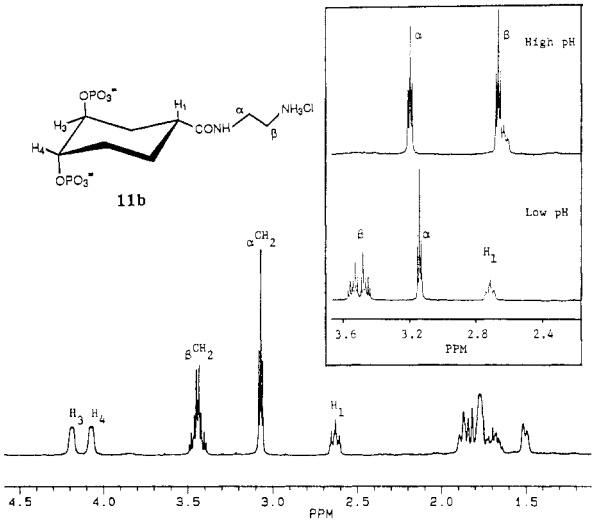

FIGURE 7: ^{1}H NMR spectrum at 500 MHz of **11b** in deuterium oxide. Characteristic signals are present for the ring and spacer arm methylene protons. The inset shows the influence of pH on the β-methylene protons of the spacer arm.

protons that shows cross-peaks to H-2 and H-5 (Figure 6). The α-methylene protons of the side chain appeared as a triplet at δ 2.85 and gave a cross-peak with a broad multiplet at δ 3.20, which was assigned to the β-methylene protons. The diaxial isomer **11b** gave two multiplets as δ 4.19 and 4.06 for the H-3 and H-4 ring protons (Figure 7). The α-methylene protons of this isomer gave a triplet at δ 3.07, while the β-methylene protons yielded a multiplet at δ 3.44. The multiplicity and downfield shift of the latter protons is attributed to a protonated terminal amino group; these protons shifted upfield and gave a characteristic triplet at δ 2.6 in alkaline solution, while the α-methylene protons remained essentially unchanged at δ 3.2 (see inset, Figure 7). Thus, at low pH the β-methylene protons are chemically nonequivalent and are deshielded by the positive charge. The H-1 ring proton for **11a** and **11b** appeared as a triplet of triplets at δ 2.15 and 2.63, respectively, with a combined coupling constant of 30 Hz, confirming its axial orientation in both compounds. The remaining protons were assigned by using two-dimensional homonuclear NMR spectroscopy (COSY 45), while ^{31}P and ^{13}C including DEPT spectra were also obtained to characterize both compounds fully.

The final step in the synthesis of the affinity column matrix (Figure 5) was achieved by coupling the ligands **11a** and **11b** to CNBr-activated Sepharose 4B according to the standard procedure. An alternative approach that involved direct coupling of the (methoxycarbonyl)cyclohexanediol bisphosphates **10a** and **10b** to a matrix containing an aminoalkyl spacer arm was not successful. The ligand was also coupled to the more hydrophilic epoxy-activated Sepharose 6B.

Intermediate **9a**, obtained during synthesis of the affinity ligand, also served as a precursor for synthesis of a radioactive IP_3 analogue for use in receptor binding studies. Reduction of the methyl ester of **9a** with sodium borotritide, in the presence of lithium bromide, gave the tritiated alcohol **13a** in 64% yield (Figure 8). A parallel synthesis of the unlabeled analogue was also undertaken to compare and monitor the reactions by TLC and NMR. As expected, the NMR spectrum of the unlabeled alcohol corresponding to **13a** indicated the presence of a methylene signal at δ 3.41 and a hydroxyl group at δ 1.62. Phosphorylation of the tritiated and unlabeled alcohols with diphenyl chlorophosphate yielded the trisphosphorylated derivative **14a**, which was characterized by

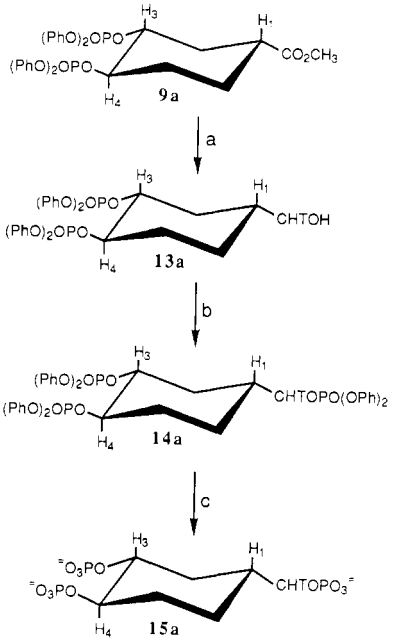

FIGURE 8: Scheme for synthesis of tritiated analogue **15a**. Reagents: (a) sodium borotritide and lithium bromide in ethanol; (b) diphenyl chlorophosphate in pyridine, 25 °C, 24 h; (c) hydrogen–platinum oxide in ethanol, 25 °C, 36 h.

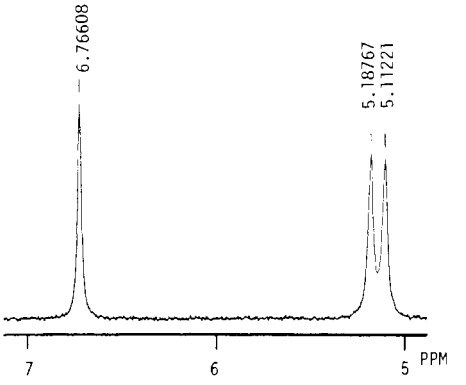

FIGURE 9: ^{31}P NMR spectrum of **15a**.

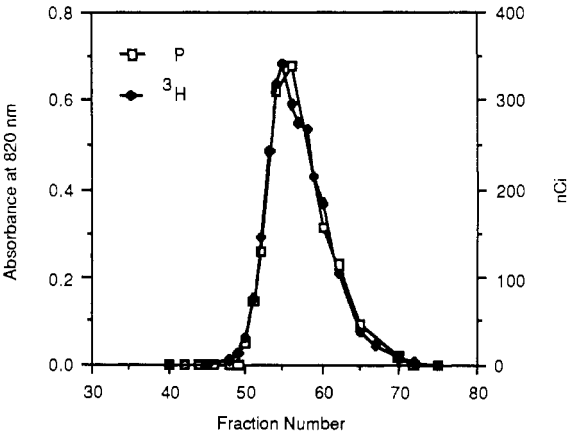

FIGURE 10: Characterization of [^{3}H]-**15a**. The radioactive derivative (◆) was coeluted with the unlabeled compound (□) on ion-exchange chromatography.

NMR spectroscopy. Catalytic hydrogenolysis of the latter with platinum dioxide, to remove the phenyl groups, furnished the desired tritiated trisphosphate, **15a**, in 35% overall yield, which was characterized by NMR (Figure 9) and by chromatographic comparison (Figure 10) with the corresponding non-

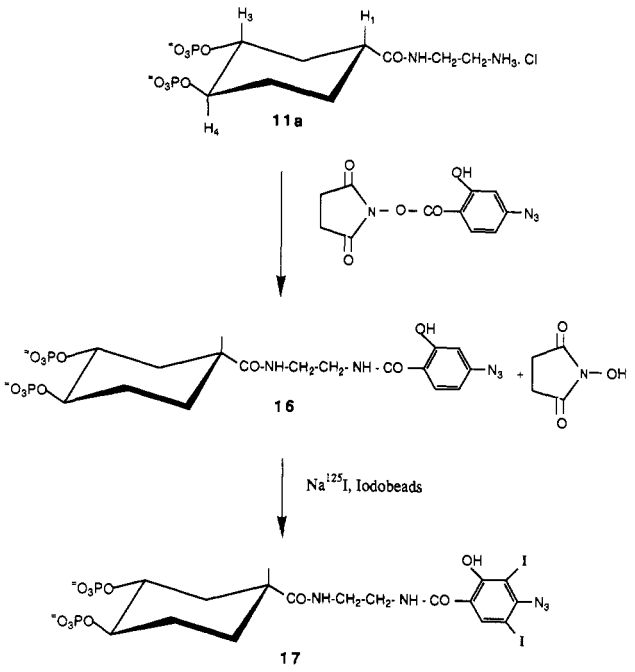

FIGURE 11: Scheme for synthesis of photoactivatable cross-linking agent.

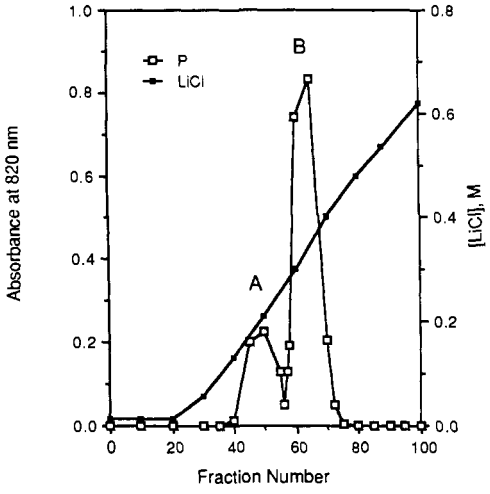

FIGURE 12: Isolation of **16** by ion-exchange chromatography. A QAE-Sephadex column was eluted with a LiCl gradient (■), and the effluent was monitored for total phosphate (□). Peak A is unreacted **11a**, and peak B is **16**.

radioactive compound.

For synthesis of a cross-linking agent that could be radioiodinated, **11a** was reacted with succinimido 4-azidosalicylate to yield the amide of 4-azidosalicylic acid (**16**, Figure 11). Removal of the side product *N*-hydroxysuccinimide proved difficult, but this was accomplished by ion exchange on a QAE-Sephadex column (Figure 12). Iodination of **16** was done with $Na^{125}I$ and Iodobeads to provide **17**, isolated by ion exchange (Figure 13), and this method proved much more convenient than the chloramine T procedure. The presence of a singlet signal for a single proton in the ^1H NMR spectrum established that diiodination had occurred. The shift in the absorption maximum from 306 to 349 nm should favor photolysis by long-wavelength UV excitation.

In addition to facilitating the identification and isolation of IP_3 binding proteins, this synthesis of affinity ligands should prove useful in unraveling the stereochemical requirements of

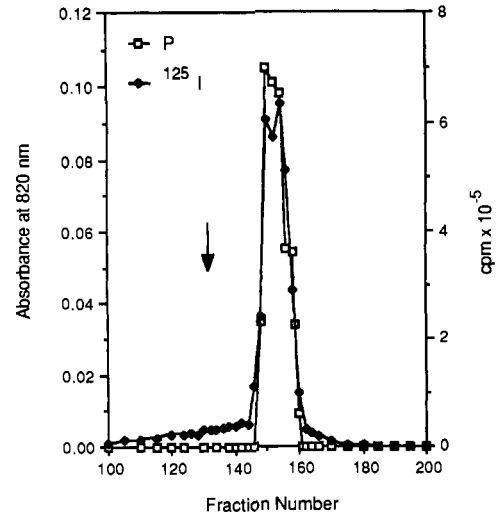

FIGURE 13: Isolation of **17** on a Sephadex G-10 column. The material applied to the column was eluted first with 0.1 M ammonium bicarbonate followed, at the arrow, with ethanol–water (1:1). A major sharp peak of ^{125}I (◆) corresponded to the single organic phosphate peak (□).

binding, particularly with regard to the orientation of the phosphate groups on positions 4 and 5 of the ring.

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