Carbohydrate Research, 234 (1992) 189–206
Elsevier Science Publishers B.V., Amsterdam

Synthesis and biological properties of 2-substituted *myo*-inositol 1,4,5-trisphosphate analogues directed toward affinity chromatography and photoaffinity labeling

Shoichiro Ozaki [a], Yutaka Watanabe [a], Tomio Ogasawara [a], Masato Hirata [b] and Takashi Kanematsu [b]

[a] *Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790 (Japan)*
[b] *Department of Biochemistry, Faculty of Dentistry, Kyushu University 61, Fukuoka 812 (Japan)*

(Received January 8th, 1992; accepted June 9th, 1992)

ABSTRACT

A series of *myo*-inositol 1,4,5-trisphosphate analogues with the 2-acyl substituents *p*-aminobenzoyl (**7**), *p*-azidobenzoyl (**8**), 4-{5-[2-(benzamido)ethyl]-2-hydroxyphenylazo}benzoyl (**9**), and *cis,trans*-4-aminocyclohexylcarbonyl (**10**) were synthesised and examined for their effects on the 5-phosphatase, the 3-kinase, the tritiated trisphosphate-binding activity, and the Ca^{2+}-releasing activity. Each analogue inhibited the hydrolysis of D-[5-^{32}P]Ins(1,4,5)P_3 and the phosphorylation of D-[^{3}H]Ins(1,4,5)P_3, catalysed by erythrocyte ghosts and brain cytosol, respectively. The analogues acted as full agonists in releasing Ca^{2+} from permeabilised cells and also inhibited the binding of D-[^{3}H]Ins(1,4,5)P_3 to cerebellum microsomes. The analogues **7** and **10** were utilised for immobilisation of the trisphosphate on Sepharose™ and the subsequent affinity chromatography effected purification of the above proteins. A photoaffinity probe, the appendage of which acted as the photoaffinity probe as well as a non-radioactive molecular marker, was also derived from the analogue **7**.

INTRODUCTION

1D-*myo*-Inositol 1,4,5-trisphosphate [D-Ins(1,4,5)P_3] is an intracellular messenger that mediates the release of Ca^{2+} from non-mitochondrial intracellular stores of calcium in stimulated cells[1]. D-Ins(1,4,5)P_3 is metabolised by an ATP-dependent phosphorylation[2] to give D-Ins(1,3,4,5)P_4 and by a 5-phosphatase-catalysed dephosphorylation[3] to give D-Ins(1,4)P_2. In order to elucidate the mechanism of the release of Ca^{2+} and metabolism at the molecular level, it is crucial to investigate

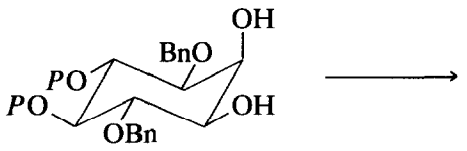
Correspondence to: Professor S. Ozaki, Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790, Japan.

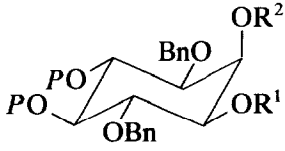
how Ins(1,4,5)P_3 interacts with the receptor protein and the metabolic enzymes, 5-phosphatase and 3-kinase.

In this context, the 2-azidobenzoyl derivative of Ins(1,4,5)P_3 was reported[4] for photoaffinity labeling and found to cause irreversible inactivation of the receptor protein for the release of Ca^{2+} under photolysis. On the basis of these findings and reports[5] that the biological activities of Ins(1,4,5)P_3 can be attributed to the 4,5-phosphate groups, whereas the 1-phosphate group increases the affinity for its recognition by the receptor site, further chemical modification of Ins(1,4,5)P_3 at position 2 has been carried out. We now describe the synthesis and biological properties of some 2-acyl derivatives and their utilisation for photoaffinity labeling and affinity chromatography.

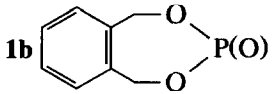
RESULTS AND DISCUSSION

Synthesis of 2-acyl-Ins(1,4,5)P_3 analogues (**7–10**).—3,6-Di-*O*-benzyl-*myo*-inositol 4,5-bis(dibenzyl phosphate) (**1a**), derived from *myo*-inositol in 5 steps, is a versatile intermediate for syntheses of *myo*-inositol polyphosphates such as the 1,2-cyclic-4,5-, and the 2,4,5- and 1,4,5-tris-phosphate[6]. Compound **1a** can also be utilised for the preparation of 2-substituted derivatives of Ins(1,4,5)P_3. The 4,5-phosphate groups are now introduced preferably by the phosphoramidite method[7] using the phosphitylating agent **2** to give **1b** instead of the method[8] employing tetrabenzyl pyrophosphate–butyl-lithium for the synthesis of **1a**. Compound **1a** was triethylsilylated selectively at HO-1 followed by nitrobenzoylation of HO-2 to afford **4**. Desilylation of **4** gave **5**, HO-1 of which was phosphorylated by successive treatment with phosphorus trichloride, benzyl alcohol, and *tert*-butyl hydroperoxide[9] to give the trisphosphate derivative **6**. Hydrogenolysis of **6** accompanied by reduction of the nitro group afforded 2-*O*-(4-aminobenzoyl)-*myo*-inositol 1,4,5-trisphosphate (**7**).





1a $P = (BnO)_2P(O)$



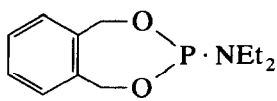
1b

3 $R^1 = Et_3Si$, $R^2 = H$

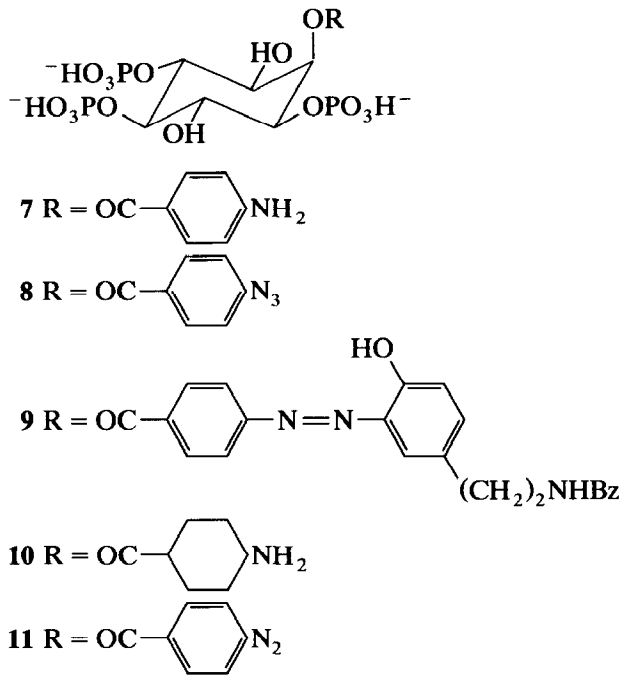
4 $R^1 = Et_3Si$, $R^2 = p\text{-}NO_2Bz$

5 $R^1 = H$, $R^2 = p\text{-}NO_2Bz$

6 $R^1 = (BnO)_2P(O)$, $R^2 = p\text{-}NO_2Bz$



2



Diazotisation of **7** followed by treatment with sodium azide and *p*-hydroxyphenylethylamine afforded the 4-azidobenzoyl derivative **8** and the diazo coupling product, benzoylation of which gave **9**. Furthermore, **7** was transformed into the cyclohexyl derivative **10** by hydrogenation at 80 atm in the presence of ruthenium(IV) oxide.

Naturally occurring Ins(1,4,5)P_3 is D, and the L form is believed to have neither physiological activity nor to interfere in the various assay systems. However, modification of the structure of D-Ins(1,4,5)P_3 may change the biological profile; hence, the enantiomers of **7** and **10** were prepared from D-**1a** and L-**1a** which were separated by chiral column chromatography using Chiralcel OD™. The analogues **7**–**10** of D-Ins(1,4,5)P_3 thus obtained were examined for their effects on the Ins(1,4,5)P_3-phosphatase, Ins(1,4,5)P_3-kinase, D-[^{3}H]Ins(1,4,5)P_3-binding activity, and Ca^{2+}-releasing activity.

Effects on D-*Ins(1,4,5)*P_3-*5-phosphatase.*—Fig. 1 shows the dose dependence of the D-Ins(1,4,5)P_3 analogues in inhibiting the hydrolysis of D-[5-^{32}P]Ins(1,4,5)P_3 by the phosphatase of human erythrocyte ghosts. Each of the racemic 2-substituted analogues **7**–**10** and the positional and cyclic isomers of $InsP_3$ dose-dependently inhibited the hydrolysis of D-[5-^{32}P]Ins(1,4,5)P_3 and was a substrate for the phosphatase, although the extents of the hydrolysis were low compared to that[10] of D-Ins(1,4,5)P_3, indicating that the analogues competed with the radioactive substrate for the active site of the phosphatase. The analogues, except for **10**, were more potent than D-Ins(1,4,5)P_3, thereby indicating that the presence of substituents or a deoxy group increased the affinity for the enzyme. Transformation of the benzene ring of **7** to the cyclohexane ring in **10** decreased the activity.

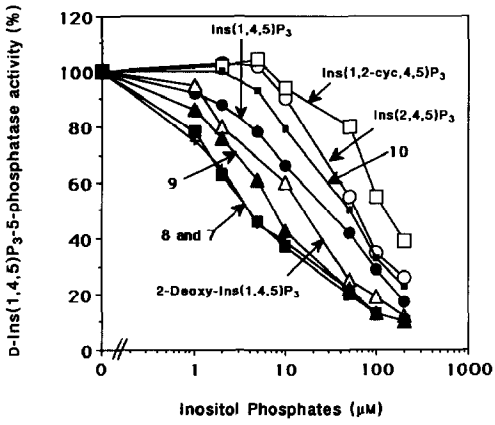


Fig. 1. Inhibition of D-[5-^{32}P]Ins(1,4,5)P_3-5-phosphatase activity by InsP_3 analogues. Erythrocyte ghosts were incubated with 10 μM D-[5-^{32}P]Ins(1,4,5)P_3 in the presence of each InsP_3 analogue at the concentrations indicated at 37° for 10 min.

In seeking to evaluate the properties of the enantiomers[11], it was found (Fig. 2) that L-**7** and L-**10** inhibited the hydrolysis of D-Ins(1,4,5)P_3 by the ghosts, whereas the D forms were inactive up to 100 μM. Moreover, L-**7** was more potent than L-**10** and D-Ins(1,4,5)P_3. Polokoff et al.[12] reported that there was no marked difference between D- and L-Ins(1,4,5)P_3 in inhibiting the phosphatase of aortic smooth muscle cells, i.e., L-Ins(1,4,5)P_3 is recognised by the active site of the phosphatase. Thus, 2-substitution of D-Ins(1,4,5)P_3 markedly reduces the affinity for the active site of the enzyme, which indicates that HO-2 is important in the recognition by the phosphatase. On the other hand, 2-substitution of L-Ins(1,4,5)P_3 increases the affinity, especially for the 4-aminobenzoyl group.

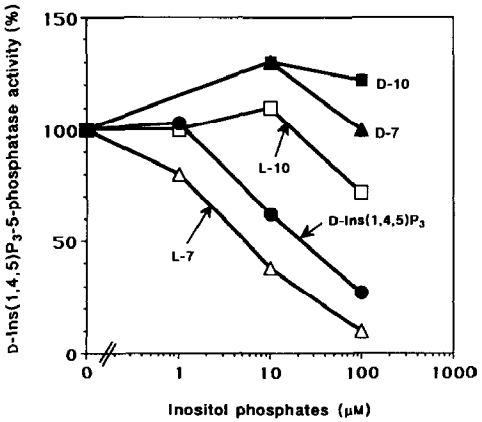


Fig. 2. Inhibition of D-[5-^{32}P]Ins(1,4,5)P_3-5-phosphatase activity by the enantiomers of the InsP_3 analogues 7 and **10**. Experimental procedures were as described in Fig. 1.

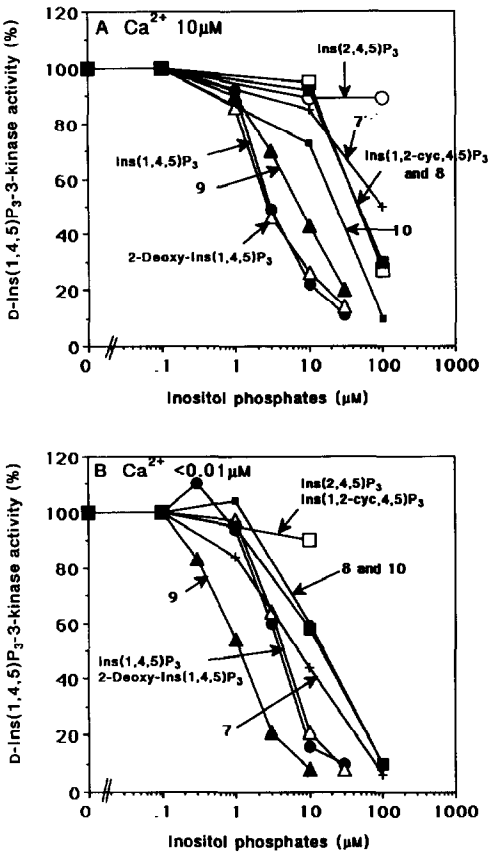


Fig. 3. Inhibition of D-[^{3}H]Ins(1,4,5)P_3-3-kinase activity by InsP_3 analogues. Rat-brain cytosol was incubated with μM D-[^{3}H]Ins(1,4,5)P_3 in the presence of each InsP_3 analogue at the concentrations indicated at 37° for 5 min and at Ca^{2+} concentrations of A, 10 μM; and B, < 0.01 μM.

Effects on D-*Ins(1,4,5)P_3-3-kinase.*—A rat-brain cytosol fraction was assayed for D-Ins(1,4,5)P_3-3-kinase in the presence of 10 mM pyrophosphate as an inhibitor of D-Ins(1,4,5)P_3-5-phosphatase. As shown in Fig. 3A, DL-2-deoxy-Ins(1,4,5)P_3 was equipotent to D-Ins(1,4,5)P_3 in inhibiting the 3-kinase in the presence of 10 μM Ca^{2+}. The other compounds, except for DL-Ins(2,4,5)P_3, were also inhibitors, although the concentrations required for half-maximal inhibition were 2–30-fold higher. At [Ca^{2+}] < 0.01 μM, however, the potency of these inhibitors was more striking, whereas those of D-Ins(1,4,5)P_3 and its 2-deoxy derivative remained unchanged (Fig. 3B).

The K_m value for D-Ins(1,4,5)P_3 for the 3-kinase was 0.8 μM, regardless of the concentration of Ca^{2+}. The K_i values, as determined by Lineweaver–Burk plots of the inhibition of the phosphorylation of D-[^{3}H]Ins(1,4,5)P_3 by the analogues, are shown in Table 1. Only **10** was a substrate for the kinase[11]. In contrast to the results with D-Ins(1,4,5)P_3-5-phosphatase, the inhibition of the 3-kinase was specific for the D isomers and the results for D-**7** and D-**10** are shown in Fig. 4. The

TABLE I

K_i values [a] for $InsP_3$ analogues in inhibiting the phosphorylation of D-[^{3}H]Ins(1,4,5)P_3

Compound	K_i	
	< 0.01 μM Ca^{2+}	10 μM Ca^{2+}
D-Ins(1,4,5)P_3	1.0	0.99
2-Deoxy-$InsP_3$	1.6	1.9
9	0.36	2.0
7	2.4	33.9

[a] The apparent K_i was determined from Lineweaver–Burk plots.

changes in the K_i values depending on [Ca^{2+}] were much the same as seen with the racemates. Thus, HO-2 of D-Ins(1,4,5)P_3 is not essential for recognition by the active site of the 3-kinase and, furthermore, the changes in conformation in or near the active site may be caused by the changes in concentration of Ca^{2+}.

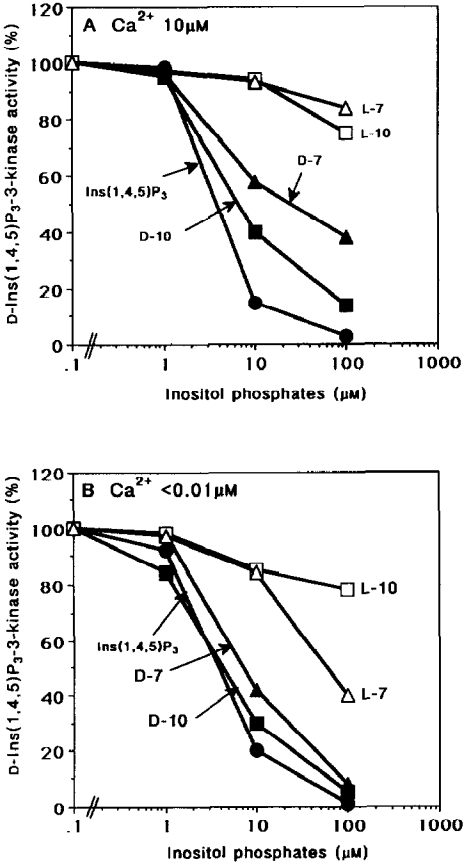


Fig. 4. Inhibition of D-[^{3}H]Ins(1,4,5)P_3-3-kinase activity by the enantiomers of the $InsP_3$ analogues **7** and **10**. Experimental procedures were as described in Fig. 3.

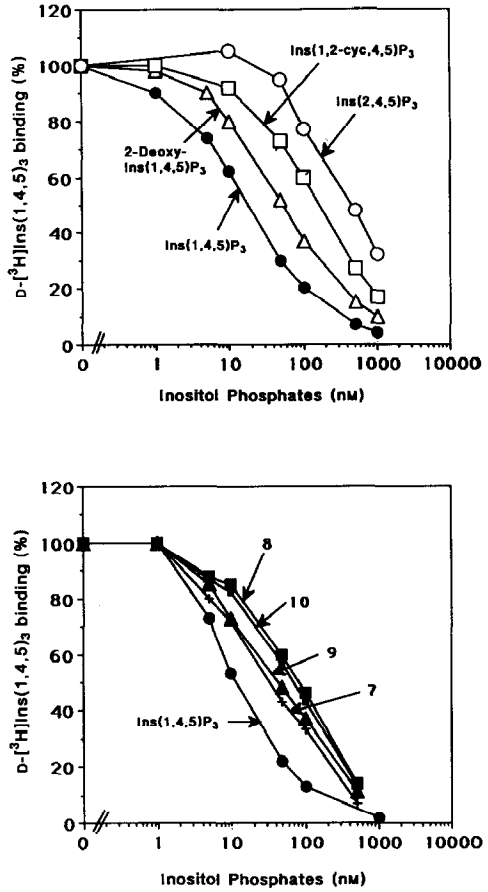


Fig. 5. Displacement of D-[^{3}H]Ins(1,4,5)P_3 from the cerebellum microsomes by InsP_3 analogues. Rat-cerebellum microsome was incubated with 0.6 nM D-[^{3}H]Ins(1,4,5)P_3 in the presence of various concentrations of each unlabeled InsP_3 analogue at 0° for 15 min.

Effects on D-*Ins(1,4,5)P_3 binding and Ca^{2+}-releasing activity.*—In order to determine the extent of competition with D-Ins(1,4,5)P_3 for the receptor site, a rat-cerebellum microsomal fraction was used. As shown in Fig. 5, each of the compounds examined could induce the dose-dependent displacement of D-[^{3}H]Ins(1,4,5)P_3 from its binding site, but with a potency less than that of D-Ins(1,4,5)P_3. The receptor for D-Ins(1,4,5)P_3 also tolerated 2-substituents, i.e., *p*-aminobenzoyl (as in **7**), *p*-azidobenzoyl (**8**), or 4-aminocyclohexylcarbonyl (**10**). These compounds were several times less potent than D-Ins(1,4,5)P_3, but the potencies of D-**7** and D-**10** were almost equal to that of D-Ins(1,4,5)P_3 and the L isomers were inactive. Thus, HO-2 of D-Ins(1,4,5)P_3 is not involved in the recognition by the receptor site.

The Ins(1,4,5)P_3 analogues **7–10** were examined for the ability to evoke the release of Ca^{2+} from saponin-permeabilised macrophages (Fig. 6). Of the accumulated Ca^{2+} at a free Ca^{2+} concentration of 0.14 μM, 40–60% was released within

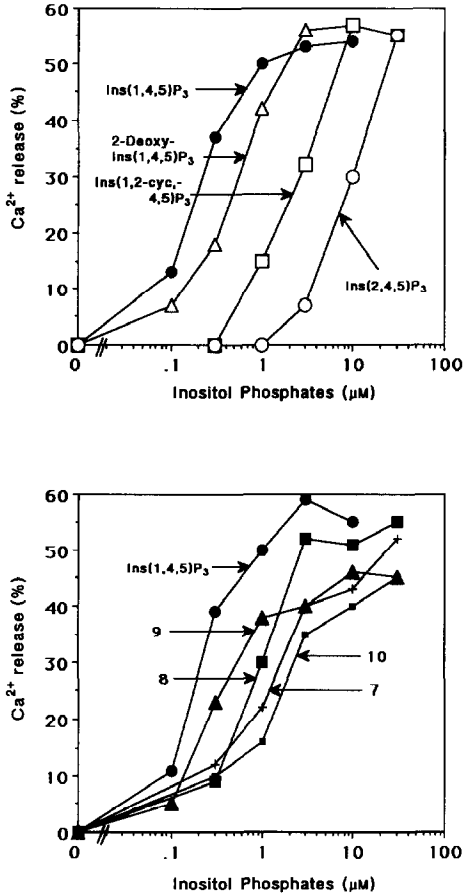
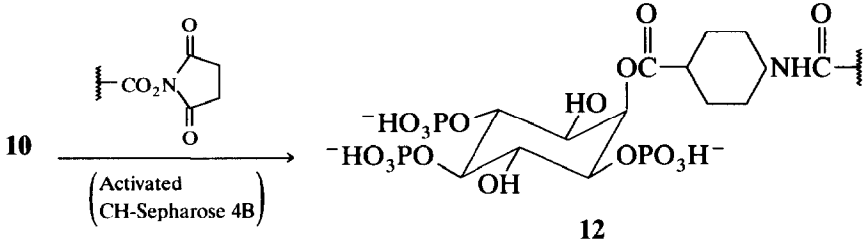


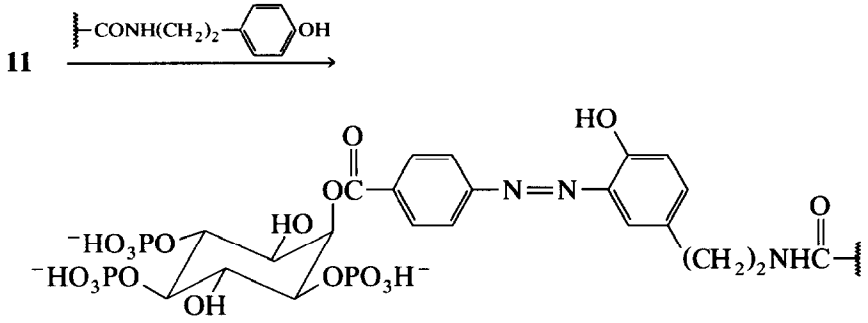
Fig. 6. Release of Ca^{2+} from permeabilised macrophages by $InsP_3$ analogues. Saponin-permeabilised macrophages were allowed to accumulate $^{45}Ca^{2+}$ in the presence of 10 mM NaN_3 at 37° for 10 min. After the Ca^{2+} accumulation reached a plateau, each $InsP_3$ analogue was added to evoke the Ca^{2+} release, and the amount of Ca^{2+} released after 1 min relative to that of Ca^{2+} accumulation was plotted against the concentration of the $InsP_3$ analogues.

1 min after adding the maximal dose. Each of the compounds tested was a full agonist and the order of potency was much the same as seen in the binding assay.

Preparation of $InsP_3$-affinity resins for chromatography and their evaluation[13].— Affinity chromatography is a useful technique for the purification of bio-molecules. As noted above, 2-substitution of Ins(1,4,5)P_3 (to give **7**–**10**) did not affect significantly the biological potency, and the analogues were full agonists. Matrices for affinity chromatography were prepared from the analogues **7** and **10**. Thus, the 2-aminocyclohexylcarbonyl derivative **10** was linked to Activated CH-Sepharose 4B™. The same gel was treated with tyramine to form the amide linkages and the resulting polymer was reacted with the diazonium salt **11**. Although the structures of these functionalised resins were not characterised fully, the analogous reactions

in solution and no recovery of each analogue in the immobilisation procedure suggest the structures **12** and **13**.





When the Ins(1,4,5)P_3-metabolising enzymes and the receptors present in the soluble and particulate fractions in rat brain were adsorbed onto the above columns and eluted by increasing salt concentrations, increased specific activities were obtained. Furthermore, novel Ins(1,4,5)P_3-binding proteins have been purified[14] from rat brain, using **13**. Thus, the Ins(1,4,5)P_3 affinity columns, especially **13**, are promising tools for purifying Ins(1,4,5)P_3-binding proteins.

Other Ins(1,4,5)P_3-affinity matrices have been reported[15,16]. Prestwich et al.[16] attached Ins(1,4,5)P_3 to Affi-Gel 10™ via the 1-phosphate group, and the resulting affinity resin was effective in the purification of Ins(1,4,5)P_3 receptor proteins.

Synthesis and biological properties of a photoaffinity probe[17].—A photoaffinity labeling technique was used to investigate the active site of enzymes. If the photoaffinity probe also has a molecular marker, then detection of the covalently bound protein is facilitated. Hirate et al.[4], Schäfer et al.[18], and Prestwich et al.[16] have prepared radioactive $InsP_3$ analogues for photoaffinity labeling, which reacted specifically with the proteins that may be involved in $InsP_3$-induced Ca^{2+} release. As a non-radioactive marker for safe handling, the Ins(1,4,5)P_3 analogue **15** was selected because it possesses a photo-sensitive azido group, and the biotinyl residue at C-2 serves as a marker based on the strong interaction with avidin and visualisation of the resulting complex[19]. Thus, N^{α}-4-azidobenzoyl-N^{ϵ}-biotinyl-L-lysine *N*-2-(4-hydroxyphenyl)ethylamide (**14**) was linked with the diazonium salt **11** generated in situ from **7** to afford the diazo coupling product **15**.

Compound **15** was shown to act as a photoaffinity probe by the following experiments. Incubation of **15** with the 5-phosphatase-rich fraction, obtained by passing red-blood-cell ghosts through a column of the Ins(1,4,5)P_3-immobilised gel **13**, followed by photolysis resulted in the specific affinity labeling of a 66-kDa protein. Moreover, addition of excess of Ins(1,4,5)P_3 to the incubation medium reduced the labeling markedly. These results indicate that **15** was photolysed at the Ins(1,4,5)P_3-recognising domain of the protein and the covalent linkage around the active site was formed. The properties of **15** are being investigated further.

EXPERIMENTAL

General methods.—When D_2O was used as a solvent for ^1H NMR spectroscopy, the signal of HOD (δ 4.64) was utilised as the reference, dioxane (δ 67.4) in a D_2O solution was used as an external reference for the ^{13}C NMR spectra, and 85% H_3PO_4 was used as the external reference for the ^{31}P NMR spectra (positive chemical shifts are downfield from the reference). The ^{13}C and ^{31}P NMR spectra were ^1H decoupled. IR spectra were recorded with a Hitachi EPI-G3 spectrometer. TLC was performed on Silica Gel 60 F254 (Merck) and cellulose (SF-2020, Funakoshi). Flash-column chromatography was performed on Wakogel C-300 and column chromatography on microgranular cellulose powder CC-31 (Whatman BioSystems). Extracts obtained after work-up were dried over $MgSO_4$ or Na_2SO_4. Experimental procedures for the preparation of the affinity resins **12** and **13** and biological experiments were reported in the articles indicated in the text.

(±)-*1,4-Di*-O-*benzyl-2,3*-O-*cyclohexylidene*-myo-*inositol 5,6-bis(dibenzyl phosphate).*—To a solution of (±)-1,4-di-*O*-benzyl-2,3-*O*-cyclohexylidene-*myo*-inositol[20] (1.86 g, 4.22 mmol) in THF (80 mL) at −78° was added butyl-lithium (5.4 mL of a 3.44 M solution in hexane, 18.57 mmol). The mixture was stirred for an additional 5 min, a solution of tetrabenzyl pyrophosphate (5.0 g, 9.29 mmol) in THF (30 mL) was added at −78°, and the mixture was stirred at 0° for 1.5 h, then filtered, and concentrated. A solution of the oily residue in ether was washed with H_2O, then dried, and the solvent was evaporated. Column chromatography (EtOAc–hexane, 1:3) of the residue on silica gel gave the title compound (3.9 g, 96%), R_F 0.7 (EtOAc–hexane, 1:1); ν_{max} 1250, 1000 cm^{-1}. NMR data ($CDCl_3$): ^1H (270 MHz), δ 1.29–1.80 (c, 10 H), 3.91 (dd, 1 H, *J* 7.9 and 3.7 Hz), 4.05 (dd, 1 H, *J* 7.3 and 6.1 Hz), 4.23 (t, 1 H, *J* 7.3 Hz), 4.33 (dd, 1 H, *J* 7.3 and 3.7 Hz), 4.62 (ddd, 1 H, *J* 12.8, 8.6, and 6.1 Hz), 4.67 (s, 2 H), 4.68 and 4.76 (ABq, 2 H, *J* 11.6 Hz), 4.80–5.10 (c, 9 H), 7.10–7.40 (c, 30 H); ^{13}C (67.8 MHz), δ 23.56, 23.88, 24.98, 33.86, 36.10, 69.07 (d, *J* 6.1 Hz), 69.21 (d, 2 C, *J* 6.1 Hz), 69.34 (d, *J* 6.1 Hz), 72.53, 72.60, 72.80 (C-2), 75.04 (C-3), 76.86 (C-1), 78.24 (t, *J* 6.1 Hz, C-4), 78.60 (C-6), 79.71 (t, *J* 6.1 Hz, C-5), 110.73, 127.37, 127.51, 127.60, 127.66, 127.73, 127.78, 127.84, 127.99, 128.05, 128.09, 128.16, 128.23, 128.27, 128.37, 135.81, 135.90, 135.94, 137.84.

Anal. Calcd for $C_{54}H_{58}O_{12}P_2$: C, 67.49; H, 6.08. Found: C, 67.82, H, 6.13.

(±)-*1,4-Di*-O-*benzyl*-myo-*inositol 5,6-bis(dibenzyl phosphate)* (**1a**).—A solution of the above derivative (1.74 g, 1.81 mmol) in trifluoroacetic acid (20 mL) and

11 + N3
CONH
CH
CONH(CH2)2
OH
(CH2)4
NH—BT
14
HO
N=N
(CH2)2NHCCH
O
NH
C
N3
(CH2)4
NH
HN
NH
H
H
S
C
O
O
OC
HO
OPO3H−
−HO3PO
−HO3PO
OH
(BT = biotinyl)
15

MeOH (19 mL) was stirred at room temperature for 2 h, then diluted with ether (100 mL). The organic solution was washed successively with aq $NaHCO_3$ (2 × 50 mL), aq $KHSO_4$ (50 mL), and brine (2 × 50 mL), dried, and concentrated. Recrystallisation of the residue from $CHCl_3$ and light petroleum (bp 30–70°) gave **1a** (1.29 g, 83%), mp 128°, R_F 0.2 (EtOAc–hexane, 1:1); ν_{max} 3450, 1236, 1120, 980 cm^{-1}. ^1H NMR data (270 MHz, $CDCl_3$): δ 3.47 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.53 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.88 (t, 1 H, *J* 9.1 Hz), 4.08 (t, 1 H, *J* 2.7 Hz), 4.52 (d, 1 H, *J* 11.1 Hz), 4.50 (q, 1 H, *J* 9.1 Hz), 4.61 (d, 1 H, *J* 11.1 Hz), 4.74 (d, 1 H, *J* 11.1 Hz), 4.85 (d, 1 H, *J* 11.1 Hz), 4.71–5.08 (c, 9 H including H-4), 7.03–7.35 (m, 30 H).

Anal. Calcd for $C_{48}H_{50}O_{12}P_2$: C, 65.44; H, 5.75. Found: C, 65.39; H, 5.73.

Resolution of **1a** *using chiral column chromatography*.—A solution of racemic **1a** (200 mg) in hexane and ^{i}PrOH (5:1) was divided into six fractions, each of which was added to a column (2 × 25 cm) of Chiralcel OD and eluted with hexane–^{i}PrOH (5:1) at 10 mL/min and 18 atm to give, first, L-**1a** (74 mg, 37%), $[\alpha]_D^{23}$ −21° (*c* 1.10, $CHCl_3$), and then D-**1a** (70 mg, 35%), $[\alpha]_D^{23}$ +21° (*c* 1.05, $CHCl_3$). Spectral data and chromatographic properties of each enantiomer were identical with those of the racemic diol.

(±)-*1,4-Di*-O-*benzyl-3*-O-*triethylsilyl*-myo-*inositol 5,6-bis(dibenzyl phosphate)* (**3**). —To a solution of **1a** (0.50 g, 0.57 mmol) in pyridine (10 mL) at 0° was added chlorotriethylsilane (0.13 g, 0.86 mmol). The mixture was stirred at room temperature for 4 h and then concentrated in vacuo. A solution of the residue in EtOAc was washed successively with H_2O (×3), satd aq $KHSO_4$ (×2), H_2O, and brine, and concentrated. Column chromatography (EtOAc–hexane, 1:3) of the residue gave **3** (0.54 g, 95%), mp 210° (from $CHCl_3$–ether), R_F 0.6 ($CHCl_3$–ether, 1:1); ν_{max} 3400, 1229, 1082 cm^{-1}. ^1H NMR data (270 MHz, $CDCl_3$): δ 0.46–0.57 (m, 6 H), 0.83 (t, 9 H, *J* 8.2 Hz), 2.40 (bs, 1 H), 3.42 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.53 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.70 (t, 1 H, *J* 2.7 Hz), 3.80 (t, 1 H, *J* 9.1 Hz), 4.4–5.1 (c, 14 H), 7.0–7.2 (c, 30 H).

Anal. Calcd for $C_{54}H_{64}O_{12}P_2Si$: C, 68.51; H, 10.82. Found: C, 68.17; H, 10.50.

(±)-*1,4-Di*-O-*benzyl-2*-O-p-*nitrobenzoyl-3*-O-*triethylsilyl*-myo-*inositol 5,6-bis(dibenzyl phosphate)* (**4**).—To a solution of **3** (1.40 g, 1.41 mmol) in pyridine (30 mL) were added *p*-nitrobenzoyl chloride (5.22 g, 28.13 mmol) and a catalytic amount of 4-dimethylaminopyridine at 0°. The mixture was stirred at room temperature for 15 h, then concentrated in vacuo, EtOAc and H_2O were added to the residue, and the organic solution was washed with H_2O, satd aq $KHSO_4$, H_2O, satd aq $NaHCO_3$, and brine, dried, and concentrated. Flash-column chromatography (EtOAc–hexane, 2:3) of the residue gave **4** (1.42 g, 88%), R_F 0.6 (EtOAc–hexane, 1:1); ν_{max}^{Nujol} 1700, 1580, 1360 cm^{-1}. ^1H NMR data (270 MHz, $CDCl_3$): δ 0.42 (m, 6 H), 0.83 (t, 9 H, *J* 8.2 Hz), 3.67 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.83 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.93 (t, 1 H, *J* 9.1 Hz), 4.5–5.1 (c, 14 H), 5.75 (t, 1 H, *J* 2.7 Hz), 7.03–7.38 (c, 30 H), 8.10 (d, 2 H, *J* 8.5 Hz), 8.25 (d, 2 H, *J* 8.5 Hz).

(±)-*1,4-Di*-O-*benzyl-2*-O-p-*nitrobenzoyl*-myo-*inositol 5,6-bis(dibenzyl phosphate)*

(**5**).—To a solution of **4** (1.4 g, 1.22 mmol) in $CHCl_3$ (3 mL) and aq 80% acetic acid (10 mL) was added *p*-toluenesulfonic acid monohydrate (0.7 g, 3.5 mmol). The solution was stirred at room temperature for 3 h, EtOAc and H_2O were added, and the organic layer was washed with aq $NaHCO_3$ and H_2O, dried, and concentrated. Column chromatography (EtOAc–hexane, 2:1) of the residue on silica gel gave **5** (1.05 g, 84%), R_F 0.5 (EtOAc–hexane, 2:1); ν_{max}^{Nujol} 3350, 1720, 1590, 1370 cm^{-1}. ^1H NMR data (270 MHz, $CDCl_3$): δ 3.70 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.80 (dd, 1 H, *J* 9.1 and 2.7 Hz), 3.90 (t, 1 H, *J* 9.1 Hz), 4.5–5.1 (c, 14 H), 5.75 (t, 1 H, *J* 2.7 Hz), 7.03–7.38 (c, 30 H), 8.10 (d, 2 H, *J* 8.5 Hz), 8.25 (d, 2 H, *J* 8.5 Hz).

(±)-*3,6-Di*-O-*benzyl*-*2*-O-p-*nitrobenzoyl*-myo-*inositol 1,4,5-tris(dibenzyl phosphate)* (**6**).—Phosphorus trichloride (248 mg, 1.8 mmol) was added dropwise to a solution of **5** (930 mg, 0.903 mmol) in THF (10 mL) and pyridine (2.5 mL) at −78°. Benzyl alcohol (584 mg, 5.4 mmol) was then added at the same temperature after 1 h, and, after stirring for 1 h, 70% *tert*-butyl hydroperoxide (464 mg, 3.6 mmol) was added. The cooling bath was removed, the mixture was stirred for 2 h, EtOAc and H_2O were added, and the organic layer was washed with aq $KHSO_4$, H_2O, aq $NaHCO_3$, and H_2O, dried, and concentrated. Flash-column chromatography (EtOAc–hexane, 1:1) of the residue gave **6** (978 mg, 83%), R_F 0.5 (EtOAc–hexane, 1:1). ^1H NMR data (270 MHz, $CDCl_3$): δ 3.70 (dd, 1 H, *J* 9.8 and 2.4 Hz), 4.12 (t, 1 H, *J* 9.5 Hz), 4.4–5.3 (c, 19 H), 6.19 (t, 1 H, *J* 2.4 Hz), 7.0–7.4 (c, 40 H), 8.10 (d, 2 H, *J* 8.6 Hz), 8.25 (d, 2 H, *J* 8.6 Hz).

(±)-*2*-O-p-*Aminobenzoyl*-myo-*inositol 1,4,5-trisphosphate* (**7**).—A mixture of **6** (0.9 g, 0.69 mmol), 5% Pd/C (1.0 g), and aq 20% MeOH (25 mL) was stirred under H_2 at room temperature for 12 h, then filtered, and concentrated. Column chromatography (aq 28% NH_4OH–^{n}PrOH–H_2O, 4:5:1), with the application of pressure, gave **7** (0.44 g, presumably the triammonium salt) which was converted into the trisodium salt by passing sequentially through columns of cation-exchange resins (pyridinium form and then the Na^+ form), mp > 280°, R_F 0.35 (aq 28% NH_4OH–^{n}PrOH–H_2O, 4:5:1); ν_{max}^{KBr} 3400, 1680, 1280, 1170, 1040 cm^{-1}. NMR data: ^1H (270 MHz, 21 mg in 0.73 mL of D_2O, neutral), δ 3.84 (dd, 1 H, *J* 9.5 and 2.8 Hz), 3.92 (t, 1 H, *J* 9.5 Hz), 4.00 (q, 1 H, *J* 9.5 Hz), 4.08 (dt, 1 H, *J* 9.5 and 2.8 Hz), 4.29 (q, 1 H, *J* 9.5 Hz), 5.65 (t, 1 H, *J* 2.8 Hz), 6.68 (d, 2 H, *J* 8.5 Hz), 7.75 (d, 2 H, *J* 8.5 Hz); ^{13}C (67.8 MHz, ~ 70 mg in 0.75 mL of D_2O, neutral), δ 70.61 (s, C-3), 72.54 (m, C-6), 73.35 (s, C-2), 73.39 (d, *J* 5.5 Hz, C-1), 77.04 (m, C-4), 78.57 (m, C-5), 115.14, 118.23, 132.44, 153.14, 168.28; ^{31}P (109 MHz, 21 mg in 2.5 mL of D_2O, pD 6.8), δ 2.11, 2.49, 3.80. FAB-mass spectrum: *m/z* 606 [M^+ (trisodium salt) + 1].

Each enantiomer was prepared in a similar manner from the corresponding optically active diol **1a** and showed identical chromatographic and spectroscopic properties: D-**7** (ammonium salt), $[\alpha]_D^{23}$ −3.6° (*c* 2.2, D_2O, neutral), L-**7** (ammonium salt), $[\alpha]_D^{23}$ + 4.2° (*c* 2.1, D_2O, neutral).

(±)-*2*-O-p-*Azidobenzoyl*-myo-*inositol 1,4,5-trisphosphate* (**8**).—To a solution of **7** (ammonium salt, 30 mg, 0.051 mmol) in concd. HCl (0.1 mL) and H_2O (3 mL) at 0°

was added a solution of $NaNO_2$ (10 mg) in H_2O (0.5 mL). The mixture was stirred for 1 h, NaN_3 (7 mg, 0.102 mmol) was added, stirring was continued for 1 h, and the mixture was concentrated under high vacuum. Column chromatography (cellulose, aq 28% NH_4OH–^{i}PrOH–H_2O, 4:5:1) of the residue gave **8** (30 mg, 95%, as the triammonium salt), R_F 0.40 (aq 28% NH_4OH–^{i}PrOH–H_2O, 4:5:1); ν_{max}^{KBr} 3200, 2100, 1260, 1100 cm^{-1}. NMR data: ^1H (270 MHz, D_2O, 20 mg in 0.76 mL, neutral), δ 3.72 (bdd, 1 H, *J* 9.1 and ~2.5 Hz), 3.82 (c, 2 H), 3.90 (m, 1 H), 4.20 (m, 1 H), 5.60 (bt, 1 H, *J* ~2.5 Hz), 7.00 (d, 2 H, *J* 8.6 Hz), 7.90 (d, 2 H, *J* 8.6 Hz); ^{13}C (67.8 MHz, 20.6 mg in 0.71 mL of D_2O, pD 6.24), δ 70.80 (s, C-3), 72.77 (m, C-6), 73.76 (d, *J* 4.9 Hz, C-1), 74.26 (C-2), 77.44 (m, C-4), 78.90 (m, C-5), 119.84, 126.10, 132.56, 146.33, 167.79; ^{31}P (109 MHz, 20.6 mg in 2.6 mL of D_2O, pD 6.6), δ 1.76, 2.55, 3.57.

(±)-*2*-O-*(4-{5-[2-(Benzamido)ethyl]-2-hydroxyphenylazo}benzoyl)*-myo-*inositol 1,4,5-trisphosphate* (**9**).—A solution of the diazonium salt **11**, prepared from **7** (30 mg, 0.051 mmol) as described above, was made alkaline to litmus with 0.1 M $NaHCO_3$ and treated with 2-(4-hydroxyphenyl)ethylammonium chloride (13.2 mg, 0.077 mmol) for 2 h. After extraction of the excess of the starting amine with $CHCl_3$, the aqueous solution was concentrated under high vacuum, and the residue was passed through a column of cellulose (aq 28% NH_4OH–^{i}PrOH–H_2O, 3:7:1). A solution of the product in H_2O and DMF (~2:1, 2 mL) was then reacted with *N*-benzoyloxysuccinimide (26 mg, 0.117 mmol) for 2 h at room temperature. Column chromatography (cellulose, aq 28% NH_4OH–^{i}PrOH–H_2O, 1:5:1) of the concentrated mixture gave **9** (7 mg, 16%), R_F 0.65 (aq 28% NH_4OH–^{i}PrOH–H_2O, 1:5:1). ^1H NMR data (270 MHz, 7.0 mg in 0.75 mL of D_2O, neutral): δ 2.79 (bt, 2 H, *J* unreadable), 3.50 (bt, 2 H, *J* unreadable), 3.89–4.71 (c, 5 H), 5.78 (bt, *J* unreadable, 1 H), 6.85 (d, 1 H, *J* 7.9 Hz), 7.25 (m, 2 H), 7.26 (d, 1 H, *J* 7.9 Hz), 7.32 (t, 1 H, *J* 7.3 Hz), 7.44 (d, 2 H, *J* 7.3 Hz), 7.54 (s, 1 H), 7.71 (d, 2 H, *J* 8.6 Hz), 8.07 (d, 2 H, *J* 8.6 Hz).

(±)-*2*-O-*4-Aminocyclohexylcarbonyl*-myo-*inositol 1,4,5-trisphosphate* (**10**).—A mixture of **7** (ammonium salt, 20 mg, 0.033 mmol), RuO_2 (20 mg), and H_2O (5 mL) was heated at 60° under H_2 (80 atm) for 3 h, then filtered, and concentrated in vacuo. The residue was neutralised by passing through a column of cation-exchange (H^+) resin, and the eluate was concentrated after the addition of pyridine and passed through a column of Dowex 50W-X2 (Na^+) resin to afford **10** (18 mg, 90%, as the trisodium salt), R_F 0.37 (aq 28% NH_4OH–^{n}PrOH–H_2O, 4:5:1); ν_{max}^{KBr} 3150, 1710, 1120, 1050 cm^{-1}. NMR data: ^1H (270 NHz, D_2O, 18 mg in 0.73 mL, neutral), δ 1.50 (b, 4 H), 1.78 (b, 2 H), 2.10 (b, 2 H), 2.75 (b, 1 H), 3.10 (b, 1 H), 3.8–4.2 (c, 5 H), 5.54 (t, 1 H, *J* 2.8 Hz); ^{13}C (67.8 MHz, 38.6 mg in 0.73 mL of D_2O, pD 6.1), δ 27.51, 27.64, 29.13, 31.28, 39.13, 49.94, 70.58 (C-3, cis), 71.46 (C-3, trans), 71.59 (C-2, trans), 72.27 (m, C-6, trans), 72.96 (m, C-6, cis), 73.38 (d, *J* 5.49 Hz, C-1, cis), 73.64 (C-2, cis), 75.38 (d, *J* 5.49 Hz, C-1, trans), 76.90 (C-4), 78.61 (m, C-5, cis), 78.85 (m, C-5, trans), 176.73 (cis), 185.49 (trans); ^{31}P (109 MHz, 38.6 mg

in 2.7 mL of D_2O, pD 6.5), cis derivative; δ 1.22, 2.24, 3.69; trans derivative; δ 1.49, 2.39, 3.37. FAB mass spectrum: *m/z* 612 [M^+(trisodium salt) + 1].

Each enantiomer was prepared in a similar manner from the corresponding optically active **7**: D-**10** (sodium salt) had $[\alpha]_D^{23}$ −4.9° (*c* 0.92, H_2O, neutral); L-**10** (sodium salt) had $[\alpha]_D^{23}$ +5.6° (*c* 0.53, H_2O, neutral).

Synthesis of N$^{\alpha}$*-4-azidobenzoyl-*N$^{\epsilon}$*-biotinyl-*L*-lysine* N*-2-(4-hydroxyphenyl)ethylamide* (**14**).—A mixture of *N*$^{\epsilon}$*-tert*-butoxycarbonyl-L-lysine (170 mg, 0.69 mmol) and *N*-4-azidobenzoyloxysuccinimide (180 mg, 0.69 mmol) in DMF (2 mL) was stirred at room temperature for 6 h and the solvent was removed in vacuo. A solution of the residue in $CHCl_3$ was treated with aq 10% $NaHCO_3$, and the aqueous layer was acidified with M HCl and extracted with $CHCl_3$. The combined $CHCl_3$ solutions were washed with H_2O, dried, and concentrated. Column chromatography (CH_2Cl_2–MeOH, 10:1) of the residue on silica gel gave the carboxylic acid (250 mg, 93%), R_F 0.3 ($CHCl_3$–MeOH, 5:1). ^1H NMR data (270 MHz, $CDCl_3$): δ 1.20–1.62 (c, 4 H), 1.38 (s, 9 H), 1.87 (b, 2 H), 3.08 (b, 2 H), 4.70 (b, 1 H), 6.90 (d, 2 H, *J* 8.6 Hz), 7.55 (b, 1 H), 7.80 (d, 2 H, *J* 8.6 Hz), 7.80 (b, 1 H).

A solution of the acid (114 mg, 0.29 mmol) in DMF (2 mL) was treated with *N*-hydroxysuccinimide (37 mg, 0.32 mmol) and dicyclohexylcarbodiimide (62 mg, 0.30 mmol) at room temperature for 4 h. The usual work-up procedure gave the corresponding succinimido ester (140 mg, 0.29 mmol). A mixture of the ester, 2-(4-hydroxyphenyl)ethylammonium chloride (50 mg, 0.29 mmol), Et_3N (35 mg, 34 mmol), and CH_2Cl_2 (2 mL) was stirred at room temperature for 3 h, then diluted with CH_2Cl_2, washed with H_2O, aq 10% oxalic acid, H_2O, aq 10% $NaHCO_3$, and H_2O, dried, and concentrated. Column chromatography (acetone–CH_2Cl_2–ether, 1:5:1) of the residue on silica gel afforded the amide (85 mg, 60%) as an amorphous solid, R_F 0.3 (acetone–CH_2Cl_2–ether, 1:5:1); $[\alpha]_D^{20}$ −4.4° (*c* 1.13, $CHCl_3$); ν_{max}^{Nujol} 3300, 2100, 1680, 1630 cm^{-1}. NMR data ($CDCl_3$): ^1H (270 MHz), δ 1.00–1.80 (c, 6 H), 1.36 (s, 9 H), 2.66 (b, 1 H), 2.75 (b, 1 H), 2.95 (b, 2 H), 3.35 (b, 1 H), 3.64 (b, 1 H), 4.53 (td, 1 H, *J* 7.3 and 6.4 Hz), 6.76 (d, 2 H, *J* 8.5 Hz), 6.76 (b, 1 H), 6.98 (d, 2 H, *J* 8.5 Hz), 7.02 (d, 2 H, *J* 8.5 Hz), 7.32 (d, 1 H, *J* 7.6 Hz), 7.79 (d, 2 H, *J* 8.5 Hz); ^{13}C (67.9 MHz), δ 22.34, 28.39, 29.82, 32.50, 34.28, 40.27, 40.61, 53.51, 79.64, 115.68, 118.93, 128.98, 129.55, 129.65, 129.96, 143.65, 155.25, 156.53, 166.31, 171.67. DI-mass spectrum: *m/z* 510 (M^+).

Anal. Calcd for $C_{26}H_{34}N_6O_5 \cdot 0.33\ H_2O$: C, 60.46; H, 6.76; N, 16.26. Found: C, 60.31; H, 6.81; N, 16.30.

A solution of the amide (126 mg, 0.25 mmol) in CH_2Cl_2 (1.5 mL) at 0° was treated with methyl phenyl sulfide (0.5 mL) and a 20% solution (0.3 mL) of trifluoroacetic acid in CH_2Cl_2. The mixture was stirred at room temperature for 12 h, then concentrated under high vacuum. To a solution of the residue in DMF (2 mL) were added the succinimido ester of biotin (85 mg, 0.25 mmol) and Et_3N (0.3 mL). After 12 h at room temperature, the volatile materials were distilled off in vacuo. The residue was washed successively with $CHCl_3$ and H_2O, and then recrystallised from MeOH and H_2O to give **14** (113 mg, 71%), mp 111–113° (dec),

R_F 0.35 ($CHCl_3$–MeOH, 7:1), $[\alpha]_D^{20}$ +24° (*c* 1.0, MeOH); ν_{max}^{Nujol} 3270, 2100, 1680, 1610 cm^{-1}. NMR data (270 MHz): ^1H ($CDCl_3$), δ 1.25–1.80 (c, 12 H), 2.16 (t, 2 H, *J* 7.2 Hz), 2.69 (c, 3 H), 2.90 (dd, 1 H, *J* 5.2 and 4.6 Hz), 3.16–3.36 (c, 3 H), 4.27 (b, 1 H), 4.44 (c, 2 H), 6.65 (d, 2 H, *J* 8.6 Hz), 7.01 (d, 2 H, *J* 8.6 Hz), 7.15 (d, 2 H, *J* 8.6 Hz), 7.89 (d, 2 H, *J* 8.6 Hz); ^{13}C (67.9 MHz), δ 25.03, 27.64, 30.18, 30.51, 30.84, 33.56, 36.36, 37.57, 40.74, 41.83, 42.95, 56.26, 57.76, 62.36, 64.10, 117.04, 120.76, 131.31, 131.61, 131.85, 132.44, 145.84, 157.63, 168.35, 169.94, 175.12, 176.79. FAB-mass spectrum: *m/z* 637 [M^+ + 1].

Anal. Calcd for $C_{31}H_{40}N_8O_5S \cdot 2\ H_2O$: C, 55.34; H, 6.59; N, 16.65. Found: C, 55.77; H, 6.39; N, 16.22.

The preparation of **15**, by diazocoupling of **14** with **12**, and its physical data have been described[17]. FAB-mass spectrum (negative): *m/z* 1253 [(M^- − 1) + 2].

Biological assays of Ins(1,4,5)P_3 analogues (**7–10**).—Biological assays of Ins(1,4,5)P_3 analogues **7–10** were performed as described previously[10,11]. Briefly, erythrocyte ghosts were prepared from human blood and assayed for Ins(1,4,5)P_3-5-phosphatase. The enzyme activity was measured, using 50 μM D-[5-^{32}P]Ins(1,4,5)P_3 as a substrate, by determining the production of ^{32}Pi radioactivity in the upper phase following phase separation by adding ammonium molybdate and organic solvent. Rat-brain cytosol fraction was assayed for D-Ins(1,4,5)P_3-kinase in the presence of 10 mM pyrophosphate as an inhibitor of D-Ins(1,4,5)P_3-5-phosphatase. The enzyme activity was determined by measuring the production of D-[^{3}H]Ins(1,3,4,5)P_4 from μM D-[^{3}H]Ins(1,4,5)P_3, which could be separated from each other by HPLC on a SAX column. Synthetic analogues **7–10** were examined as inhibitors of the kinase activity. D-[^{3}H]Ins(1,4,5)P_3 binding to rat cerebellar microsomes was assayed in the presence or absence of various doses of Ins(1,4,5)P_3 analogues at 0.6 nM D-[^{3}H]Ins(1,4,5)P_3 by the filtration method. The Ins(1,4,5)P_3 analogues **7–10** were also examined for the ability to evoke the release of Ca^{2+} from saponin-permeabilised macrophages. Saponin-permeabilised cells accumulated $^{45}Ca^{2+}$ at a free $^{45}Ca^{2+}$ concentration of 0.14 μM. When the $^{45}Ca^{2+}$ accumulation reached a plateau level, various doses of the analogues were added to release the $^{45}Ca^{2+}$.

Preparation of affinity resins (**12, 13**) *and their evaluation in biological systems.*—Affinity resins **12** and **13** were prepared according to the method described [13]. The rat-brain cytosol or detergent extract of the membrane fraction was applied to the affinity column equilibrated with 0.05 M KCl solution, and the adsorbed proteins were eluted by a stepwise increase in salt concentration (0.2, 0.5, and 2 M KCl). The 3 fractions were assayed for Ins(1,4,5)P_3-5-phosphatase and the 3-kinase in the cytosol fraction or the phosphatase and Ins(1,4,5)P_3-binding in the detergent extract, respectively. For example, Ins(1,4,5)P_3-phosphatase activities in both cytosol and detergent-extract fractions were eluted from the column of **13** by a 0.5 M KCl solution, providing a 3 to 5-fold increase in the specific activity; in the case of the column of **12**, the activities were eluted by 0.05 M KCl and did not change before and after the chromatography. Ins(1,4,5)P_3-3-kinase or Ins(1,4,5)P_3-binding

activity was eluted from each column by 2 M KCl, giving a 20-fold or 100-fold increased specific activity, respectively.

Photoaffinity labeling with **15**.—The protein sample (~ 0.5 mg; a 5-phosphatase-rich fraction obtained by affinity chromatography of erythrocyte ghosts, using the column mentioned above) was incubated in 30 μL of a solution containing 50 mM Tris–HCl buffer (pH 8.3), 30 mM NaCl, mM EDTA, and various concentrations of **15**, with or without a 100-fold excess of Ins(1,4,5)P_3, on ice for 5 min, and the mixture was photolysed on ice under a Toshiba FL-20E lamp at a distance of 5 cm for 10 min. Following SDS–polyacrylamide gel electrophoresis and transfer to a nitrocellulose sheet, the sheet was immersed in a solution containing 5% (w/v) of dry milk in Tris-buffered saline (TBS: 0.15 M NaCl and 10 mM Tris–HCl buffer, pH 7.5) for 30 min. After washing twice with TBS, the sheet was incubated for 30 min with the streptavidin–alkaline phosphatase conjugate diluted 3000-fold with TBS. Finally, a mixture of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and Nitro Blue Tetrazolium in DMF was added to observe the labeling.

ACKNOWLEDGMENTS

This work was supported financially by The Mochida Memorial Foundation for Medical and Pharmaceutical Research (M.H.) and by a Grant-in-Aid for Scientific Research (S.O., Y.W., and M.H.) from the Ministry of Education, Science and Culture of Japan).

REFERENCES

1 M.J. Berridge and R.F. Irvine, *Nature (London)*, 312 (1984) 315–321; M.J. Berridge, *Annu. Rev. Biochem.*, 56 (1987) 159–193; J.R. Williamson, R.H. Cooper, S.K. Joseph, and S.P. Thomas, *Am. J. Physiol.*, 248 (1985) c203–c216.
2 R.F. Irvine, A.J. Letcher, J.P. Heslop, and M.J. Berridge, *Nature (London)*, 320 (1986) 631–634.
3 P.W. Majerus, T.M. Connolly, V.S. Bansal, R.C. Inhorn, T.S. Ross, and D.L. Lip, *J. Biol. Chem.*, 263 (1988) 3051–3054.
4 M. Hirata, T. Sasaguri, T. Hamachi, T. Hashimoto, M. Kukita, and T. Koga, *Nature (London)*, 317 (1985) 723–725.
5 R.F. Irvine, R.D. Brown, and M.J. Berridge, *Biochem. J.*, 222 (1984) 269–272; G.M. Burgess, R.F. Irvine, M.J. Berridge, J.S. McKinney, and J.W. Putney, Jr., *ibid.*, 224 (1984) 741–746.
6 Y. Watanabe, T. Ogasawara, H. Nakahira, T. Matsuki, and S. Ozaki, *Tetrahedron Lett.*, 29 (1988) 5259–5262.
7 Y. Watanabe, Y. Komoda, K. Ebisuya, and S. Ozaki, *Tetrahedron Lett.*, 31 (1990) 255–256.
8 Y. Watanabe, H. Nakahira, M. Bunya, and S. Ozaki, *Tetrahedron Lett.*, 28 (1987) 4179–4180.
9 Y. Watanabe, T. Ogasawara, N. Shiotani, and S. Ozaki, *Tetrahedron Lett.*, 28 (1987) 2607–2610.
10 M. Hirata, Y. Watanabe, T. Ishimatsu, T. Ikebe, Y. Kimura, K. Yamaguchi, S. Ozaki, and T. Koga, *J. Biol. Chem.*, 264 (1989) 20303–20308.
11 M. Hirata, F. Yanaga, T. Koga, T. Ogasawara, Y. Watanabe, and S. Ozaki, *J. Biol. Chem.*, 265 (1990) 8404–8407.
12 M.A. Polokoff, G.H. Bencen, J.P. Vacca, S.J. deSolms, S.D. Young, and J.R. Huff, *J. Biol. Chem.*, 269 (1989) 11922–11927.
13 M. Hirata, Y. Watanabe, T. Ishimatsu, F. Yanaga, T. Koga, and S. Ozaki, *Biochem. Biophys. Res. Commun.*, 168 (1990) 379–386.

14 T. Kanematsu, H. Takeya, Y. Watanabe, S. Ozaki, M. Yosida, T. Koga, S. Iwanaga, and M. Hirata, *J. Biol. Chem.*, 267 (1992) 6518–6525.
15 A.N. Jina, J. Ralph, and C.E. Ballou, *Biochemistry*, 29 (1990) 5203–5209.
16 G.D. Prestwich, J.F. Marecek, R.J. Mourey, A.B. Theibert, C.D. Ferris, S.K. Danoff, and S.H. Snyder, *J. Am. Chem. Soc.*, 113 (1991) 1822–1825; V.A. Estevez and G.D. Prestwich, *Tetrahedron Lett.*, 32 (1991) 1623–1626; J.F. Marecek and G.D. Prestwich, *ibid.*, 32 (1991) 1863–1866.
17 Y. Watanabe, M. Hirata, T. Ogasawara, T. Koga, and S. Ozaki, *Bioorg. Med. Chem. Lett.*, 1 (1991) 399–402.
18 R. Schäfer, M. Nehls-Sahabandu, B. Grabowsky, M. Dehlinger-Kremer, I. Schulz, and G.W. Mayr, *Biochem. J.*, 272 (1990) 817–825; G. Reiser, R. Schäfer, F. Donie, E. Hülser, M. Nehls-Sahabandu, and G.W. Mayr, *ibid.*, 280 (1991) 533–539.
19 F. Wedekind, K.B.-Pontzen, S.B.-Mohan, D. Choli, H. Zahn, and D. Brandenburg, *Biol. Chem. Hoppe-Seyler*, 370 (1989) 251–258; F.M. Finn, C.J. Stehle, and K. Hofmann, *Biochemistry*, 24 (1985) 1960–1965.
20 A.I. Lyutik, V.N. Krylova, S.P. Kozlova, B.A. Klyashchitskii, V.I. Shvets, R.P. Evstigneeva, and E.S. Zhdanovich, *Zh. Obshch. Khim.*, 41 (1971) 2747–2753.