Touching All the Bases: Synthesis of Inositol Polyphosphate and Phosphoinositide Affinity Probes from Glucose

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Introduction

Receptor-activated cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2, or simply PIP_2) by phospholipase C (PLC) (Figure 1) 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 (IP_3) interacts stereospecifically with membrane receptors to promote the release of Ca^{2+}, a key event in cellular signal transduction.[2,3] This process mediates cellular responses to hormones, neurotransmitters, and other cellular signals including odorants[4−6] and bitter taste.[7] The interaction of individual inositol polyphosphates (IP_ns) with cellular targets has been intensely studied in the past eight years, and both the chemistry[8,9] and biochemistry of IP_ns[10−12] have been extensively reviewed.

However, the phosphosinositide (PI) pathway is not restricted to the inositol polyphosphates alone. A PI 3-kinase[13,14] is linked to protein tyrosine kinases activated by a number of peptide hormones,[15,16] and the principal reaction product appears to be PtdIns-(3,4,5)P_3, or simply PIP_3, arising from phosphorylation of PIP_2 (Figure 1). Phosphoinositide polyphosphates are important in recruitment of signaling proteins to cell membranes,[17] in modulating actin polymerization,[18] and in regulating membrane traffic.[19] Finally, the PI pathway generates (or responds to) a myriad of other inositol polyphosphates.[20] Affinity chromatography methods have been employed to separate IP_n kinase, phosphatase, and binding activities.[21−24] A selection of the most important IP_ns is shown in Figure 2, with the number of phosphates increasing from left to right.

About the IP_3 Receptor. A 260 kDa receptor protein (IP_3R) that specifically recognizes Ins(1,4,5)-P_3 and mediates its role in calcium release[25] was first shown to be localized to the endoplasmic reticulum in cerebellar neurons.[26] The Ca^{2+} channel is formed by a homotetramer of IP_3R subunits, which exist in at least three main subtypes, each with alternatively spliced forms.[10,27] Heterotetramers also form naturally and increase receptor diversity.[28,29] IP_3R undergoes phosphorylation of two serines during activation,[30] and an ATP binding site has been identified by photoaffinity labeling.[31] Ca^{2+}-dependent calmodulin binding to the receptor has been observed,[32] and the immunophilin FKBP modulates Ca^{2+} flux through the IP_3R channel.[33,34]

Glenn D. Prestwich, a *bona fide* boomer from 1948, grew up in California, upstate New York, and Maryland. Lured West, he graduated from CalTech (B.Sc., 1970) and Stanford University (Ph.D., 1974) and then headed back East to Cornell University and then to Nairobi, Kenya (NIH postdoc, 1974–1976). From organic synthesis, he moved first into insect natural products chemistry and biochemistry, and then to the applications of photoaffinity labeling in ligand–receptor interactions. He was on the faculty in the Department of Chemistry and the Department of Biochemistry & Cell Biology at the State University of New York at Stony Brook from 1977 to 1996. Recently, the metastatic proliferation of his interests in biochemistry, cell biology, and biotechnology has led him back West; he is now the Presidential Professor and Chair of Medicinal Chemistry at the University of Utah. This Account is a celebration of why organic chemistry is still the central science for much of biology.

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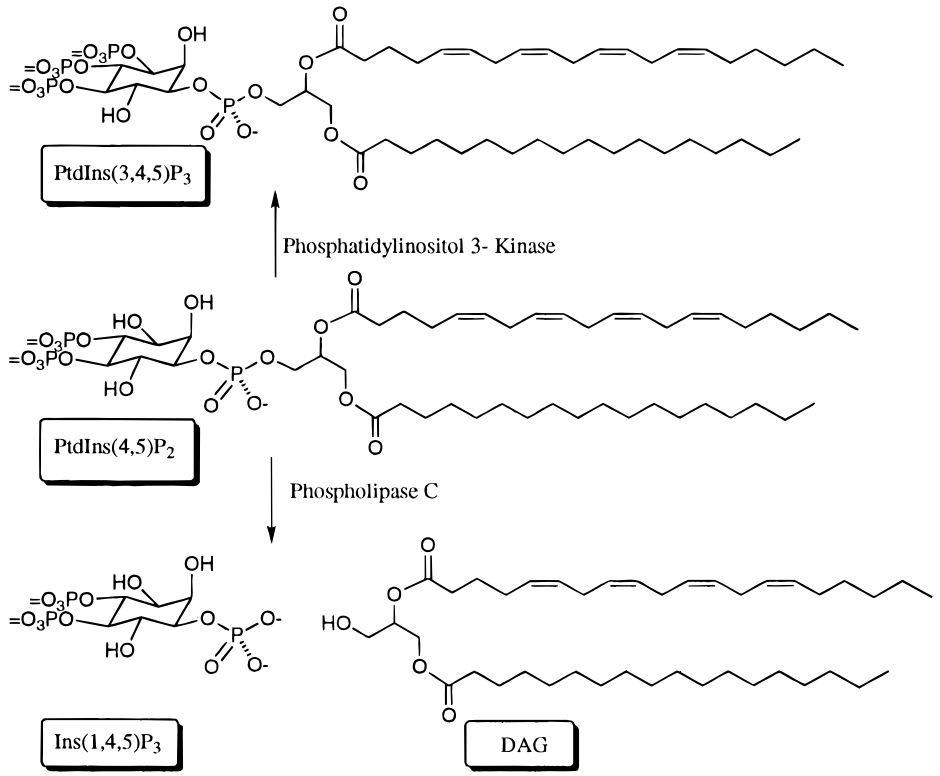

Figure 1. Phosphatidylinositol 4,5-bisphosphate is converted to three additional cell signaling molecules by phosphatidylinositol 3-kinase and phospholipase C. Note: For all phosphate monoesters, the symbol "=" signifies the presence of one or two anionic charges, protons, sodiums, or other appropriate monovalent cations, depending on the isolation procedure used and the buffer employed.

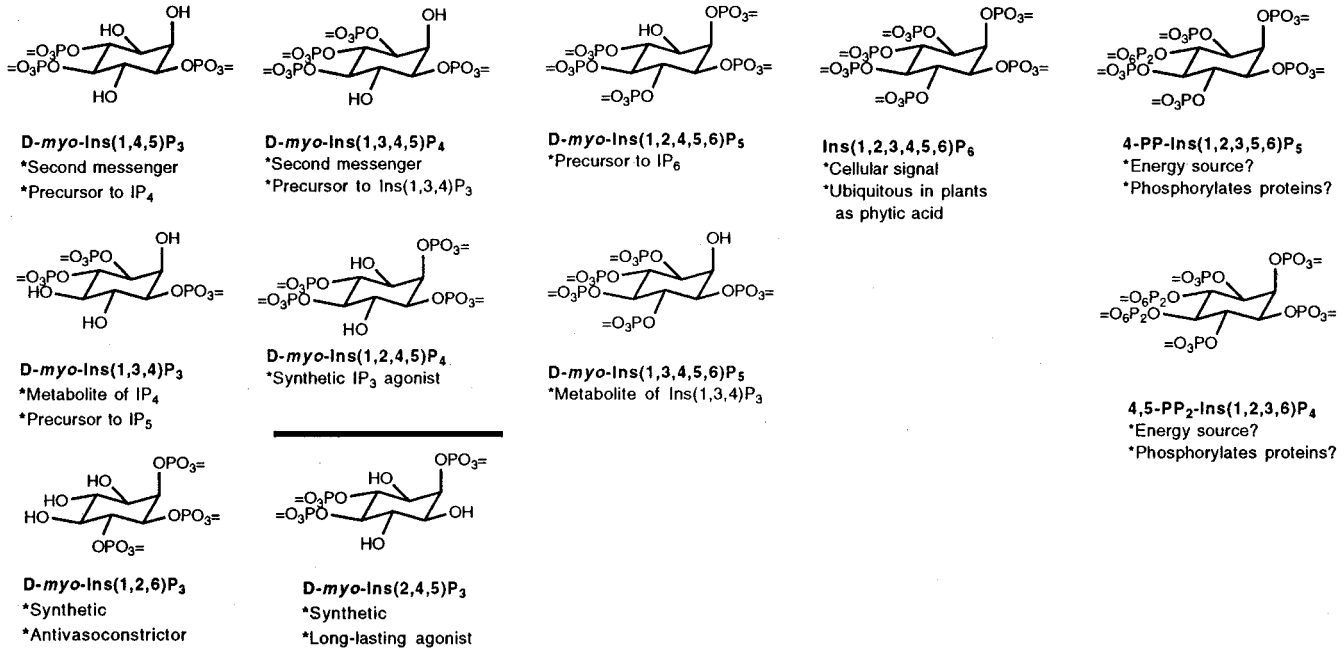

Figure 2. A selection of naturally-occurring and synthetic inositol polyphosphates.

The primary structure of the 2749-amino acid IP_3R[35,36] shows six[37] transmembrane domains at the C-terminus. A construct lacking the N-terminal 418 amino acids failed to bind IP_3, and an IP_3R (1−788) construct possessed full IP_3 binding activity.[38] The binding region for IP_3 in different IP_3R proteins was localized by neutralizing antibodies, by truncation studies,[35,38] and by photoaffinity labeling[39] to an Arg- and Lys-rich stretch near residue 475.

Rationally synthesized affinity probes for Ins(1,4,5)-P_3 were independently developed in Japan, Germany,

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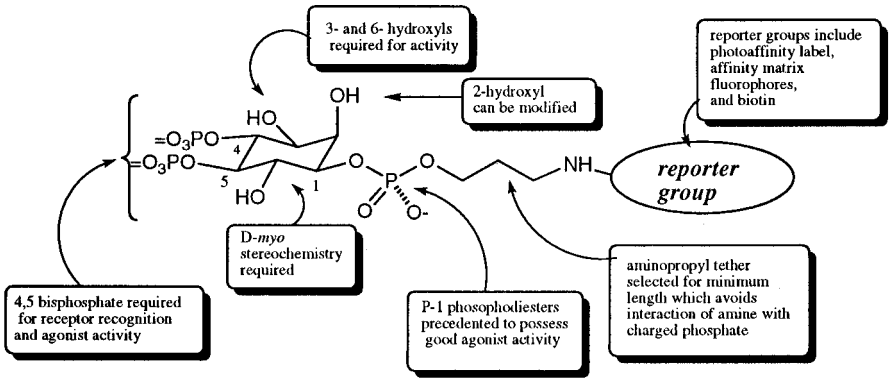

Figure 3. Design features for *myo*-Ins(1,4,5)P_3 receptor probes.

and the USA.[21,40−42] Compounds prepared by Ozaki and co-workers feature derivatization of the C-2 hydroxyl group of IP_3,[43] and these affinity ligands allowed facile purification of soluble binding proteins for IP_3.[44] Our group employed selective modification of the P-1 phosphate[45] (Figure 3) based on two precedents: (i) semisynthetic IP_3 analogs[46] that stimulated calcium release *in vitro* and (ii) photolabile P-1-modified phosphate esters of IP_3 that showed activity *prior* to photochemical deprotection.[47] The importance of the 3-OH and 6-OH groups of IP_3 in IP_3R activation of Ca^{2+} flux has been demonstrated.[48,49] The aminoethyl-tethered probes[41] showed K_D values in rat pancreatic cells 9-fold lower than that of IP_3, and a photoaffinity derivative labeled three small proteins (<49 kDa). In contrast, the 1-*O*-aminopropyl-tethered [^{125}I]-azidosalicylamide-IP_3 ([^{125}I]ASA-IP_3) and [^{3}H](4-benzoyldihydrocinnamide-IP_3 ([^{3}H]BZDC-IP_3) developed in our labs specifically labeled a >220 kDa protein, the rat brain IP_3R.[39]

Receptors for IP_4 and IP_6. Several proteins specifically recognize other inositol polyphosphates; in particular, high-affinity binding of Ins(1,3,4,5)P_4 (hereafter, IP_4) is distinct from IP_3 binding in brain tissues.[50−52] Several putative IP_4Rs have been purified by affinity chromatography (rat brain[22]) or by conventional means (pig brain[53]). The metabolism and functional roles for IP_5 or IP_6 were recently summarized[54] and include specific high-affinity binding to cerebellar membranes,[22] to pituitary gland membranes,[55,56] to specific domains in synaptic vesicle proteins,[57] to the assembly proteins[58,59] AP-2 and AP-3 important in endocytosis, to the coated vesicles in the Golgi complex,[60] and to brain and liver cytosolic[61] proteins. IP_5 and IP_6[62] can regulate IP_4 3-phosphatase activity and enhance Ca^{2+} uptake in primary cultures of brain cells.

The IP_6R isolated from the IP_4 affinity column was efficiently photoaffinity labeled by [^{125}I]ASA-IP_4 in racemic[63,64] and chiral forms.[65] The partial amino acid sequencing of this IP_6R and the sequencing of a cDNA clone showed that this protein was identical to AP-2,[59,66] one of several protein complexes associated with the formation of clathrin-coated vesicles.[67] AP-2 consists of two larger (114 and 106 kDa) and two smaller (50 and 17 kDa) subunits; AP-2 binds ATP and PI intermediates, leading to self-association.[68,69]

Development of the Ferrier Rearrangement Route to Chiral IP_n Derivatives

A poster presented by Steve Bender at a 1990 American Chemical Society meeting showed the use of a Ferrier rearrangement[70] to prepare selectively-protected Ins(1,3,4,5)P_4 derivatives from α-D-glucose. Bender's route readily allowed differentiation of the 1-phosphate from the 3,4,5-trisphosphates, thus enabling facile introduction of a linker group. Interest-

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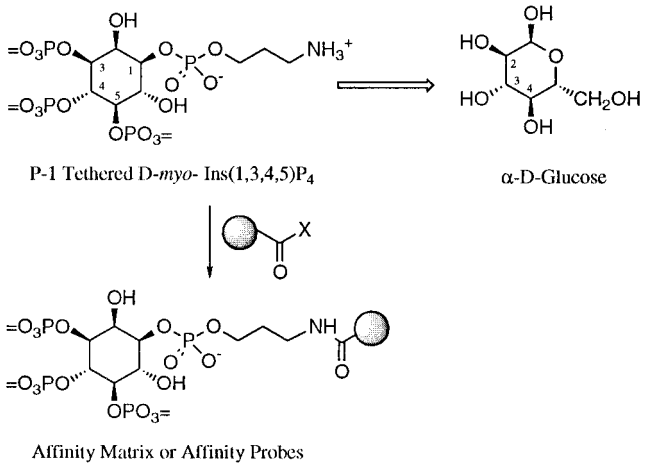

Figure 4. Retrosynthetic strategy for the Ferrier rearrangement route to *myo*-Ins(1,3,4,5)P_4.

ingly, the enzyme D-*myo*-inositol 3-phosphate synthase converts glucose-6-phosphate to the inositol 1-phosphate by a biochemical ring opening[71] followed by a stereospecific intramolecular aldol reaction[72] that is in fact the biological counterpart of the Ferrier rearrangement. We therefore switched from our racemic synthetic approaches based on inositol as a cheap starting material to a chiral synthesis based on glucose as the starting material (Figure 4). In this Account, we show how the Ferrier route can provide access to many different IP_n and PIP_n derivatives bearing tethered reporter groups.

P-1-Tethered Ins(1,3,4,5)P_4. Racemic P-1-*O*-(aminopropyl)-Ins(1,3,4,5)P_4 was first synthesized from *myo*-inositol[63] by modification of a route[21] to the corresponding Ins(1,4,5)P_3 derivative using selective protection−deprotection strategies.[8] To obtain the D-*myo* enantiomer, we employed a Ferrier-based synthesis starting with methyl α-D-glucopyranoside (**1**)[65] (Figure 5). The use of *p*-methoxybenzyl (PMB) ethers (e.g., in **2**) allowed deprotection under oxidative conditions without affecting hydrogenolytically labile groups. Thus, the key precursor **3** for the Ferrier rearrangement was prepared by tritylation, PMB etherification, detritylation, and oxidation/enol acetate formation. The Ferrier rearrangement of **3** with mercuric acetate gave inosose **4** with >90% axial 2-OH, and stereoselective reduction of this β-hydroxyketone with NaBH(OAc)$_3$ provided the key differentially-protected chiral intermediate **5** in 17% overall yield. This route has become a staple of our research effort, and has been scaled up to the multigram quantities of **5** for preparation of PIP_3 analogs (see below). The aminopropyl-IP_4 analog **7a**, obtained via hydrogenolysis of **6** followed by ion exchange purification, has now been employed to prepare biotinylated (**7c**) and fluorescent probes in addition to IP_4 affinity columns and IP_4 photoaffinity labels (**7b**).

Bender presented mechanistic studies and optimized the stereoselectivity of the Ferrier rearrangement leading to unmodified D-*myo*-Ins(1,3,4,5)P_4.[73a] The Ferrier reaction proceeded via oxymercuration to stable organomercurials that did not cyclize to product inososes until excess chloride ion was added to drive the reaction to completion. When workup was performed immediately after chloride addition, diastereomeric α-mercurio ketones were formed from the (*Z*)- and (*E*)-enol acetates. With excess chloride, *both* enol acetates appeared to give the axial C-2 OH (*myo*-inositol numbering) and equatorial C-1 OAc with strong diastereoselectivity. A comprehensive review of this reaction was recently published,[70] and a detailed mechanistic study using deuterated glucose was described.[73b] Several additional selectively P-1-modified Ins(1,3,4,5)P_4 derivatives were prepared by our route.[74]

Extension to Other IP_4 and IP_3 Derivatives

Selective manipulation of protecting groups in our original Ferrier route to Ins(1,3,4,5)P_4 has provided access to several unnatural IP_4 regioisomers. The strategy devised by Dr. G. Dormán involved first blocking the C-4 and C-6 hydroxyls of methyl α-D-glucopyranoside as the 4-methoxybenzylidene acetal (Figure 6). After protection of the two remaining OH groups, a regioselective reductive cleavage of the benzylidene acetal gave the primary alcohol at C-6 for further processing.

The developmental drug α-Trinositol, D-*myo*-Ins(1,2,6)P_3, was produced by selective degradation of phytic acid with phytase.[75] Until 1996, this compound was under development by Perstorp Pharma as a novel anti-inflammatory[76] and anti-vasoconstrictive therapeutic agent for analgesia.[77] Two chemically-modified photoaffinity probes were prepared to determine the molecular target of α-Trinositol.

Figure 7 shows the synthesis[78] of P-5-(aminopropyl)-Ins(1,2,5,6)P_4, selected on the basis of similar biological activity and binding affinity[79] of Ins(1,2,5,6)P_4 relative to α-Trinositol. Primary alcohol **8** was converted by the sequence described above to give inosose **9**. Introduction of the 1,2,6-tris(phosphomonoester) groups, PMB removal, installation of the P-5-aminopropyl linker **10**, and hydrogenolysis followed by amidation gave [^{3}H]BZDC-P-5-*O*-(aminopropyl)-D-*myo*-Ins(1,2,5,6)P_4 (**11**). Proteins in rat brain, pig aorta, or heart muscle and human umbilical vascular smooth muscle cells were labeled using photoprobe **11**. Although [^{3}H]-α-Trinositol bound to specific sites in epithelial cells of umbilical vasculature,[80] only nonspecific photolabeling was observed with **11** (A. Chaudhary, unpublished results). Binding assays with pig aorta membranes[79] using **11** as a ligand showed only low-affinity sites.

On the basis of these results and the knowledge that 3,4,5-triesters appeared to be active forms of α-Trinositol,[75] we reasoned that a 4-acylated Ins(1,2,6)P_3 might be a more suitable photoprobe. To this end, the

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(77) Wahlestedt, C.; Reis, D. J.; Yoo, H.; Adamsson, M.; Andersson, D.; Edvinsson, L. *Neurosci. Lett.* **1992**, *143*, 123−126.
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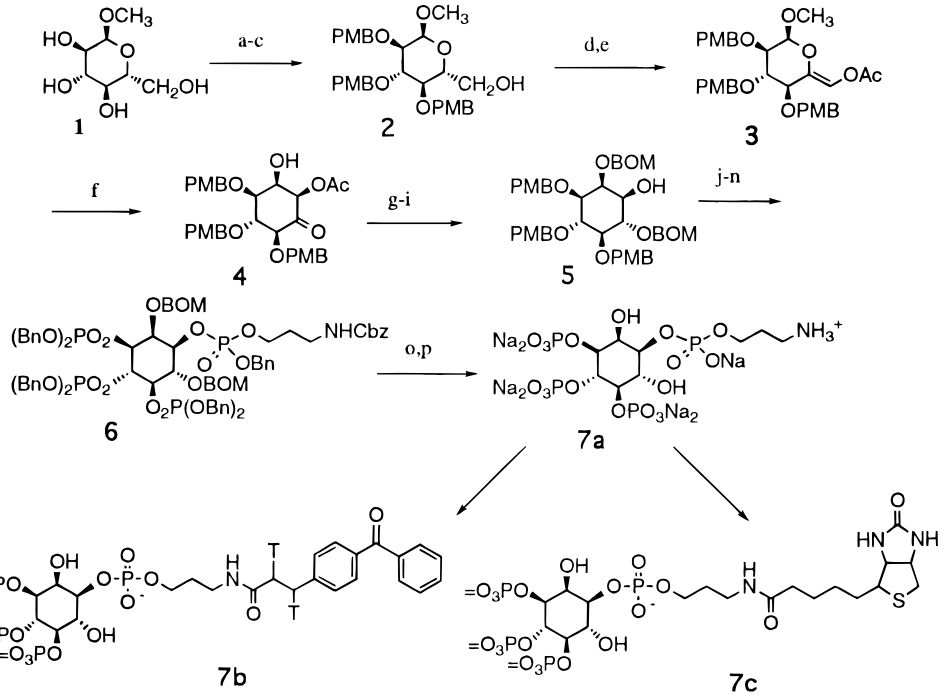

Figure 5. Ferrier route to D-*myo*-P-1-*O*-(aminopropyl)-Ins(1,3,4,5)P_4. Reagents: (a) Ph_3CCl, DMAP, Et_3N, DMF; (b) NaH, *p*-MB-Cl, DMF; (c) 5% H_2SO_4, CH_3OH; (d) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2; (e) Ac_2O, K_2CO_3; (f) $Hg(OAc)_2$, Me_2CO-H_2O; then saturated NaCl; (g) $NaBH(OAc)_3$, AcOH; (h) BOM-Cl, Bu_4NBr, H^+ sponge, CH_3CN; (i) NaOH, MeOH; (j) phosphite linker, tetrazole, CH_2Cl_2; (k) *m*-CPBA; (l) DDQ, wet CH_3CN; (m) $(BnO)_2PNiPr_2$, tetrazole, CH_2Cl_2; (n) *m*-CPBA; (o) H_2 (4 atm), Pd/C, EtOH; (p) Chelex column (Na^+ form), $Et_3NH^+HCO_3^-$ (TEAB) buffer, pH 8.3.

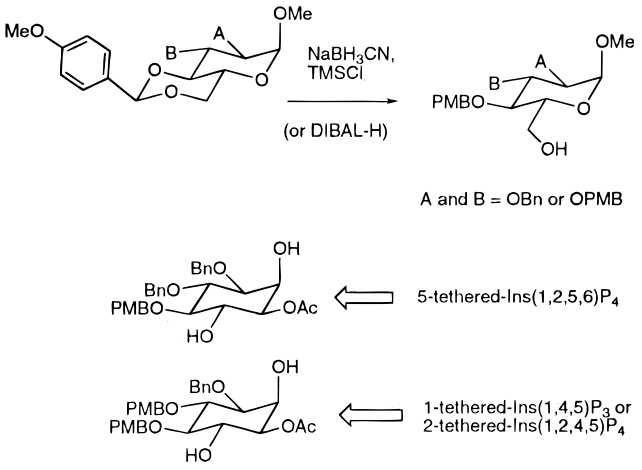

Figure 6. Benzylidene ketal approach for preparation of derivatives of Ins(1,2,5,6)P_4, Ins(1,2,4,5)P_4, and Ins(1,4,5)P_3.

4,6-benzylidene ketal of methyl α-D-glucopyranoside was opened with $(i\text{Bu})_2\text{AlH}$ to glucose derivative **12** (Figure 8); Ferrier rearrangement and selective reduction gave intermediate **13**, and manipulation of protecting groups and introduction of phosphates gave **14**. The C-4 OH was acylated to a 5-aminopentanoyl derivative, which was amidated to photoaffinity label **15**. Photoaffinity labeling experiments with the 4-acyl-[^{3}H]BZDC derivative **15** revealed selectively-labeled proteins in human platelet membranes and in human umbilical smooth muscle epithelial cells (A. Chaudhary, unpublished results).

Efforts to characterize the rat olfactory IP_3R using P-1-tethered Ins(1,4,5)P_3 affinity matrix and photoaffinity labels had been unsuccessful, despite promising results with catfish olfactory IP_3R.[81] Curiously, Ins-(2,4,5)P_3 showed 50- to 100-fold higher potency relative to Ins(1,4,5)P_3 in rat olfactory tissues in two physiological assays. In addition, several cell types exhibited Ca^{2+} release in response to D-*myo*-Ins(1,2,4,5)P_4 with the same profile expected for stimulation of the IP_3R by Ins(1,4,5)P_3.[82,83] A hybrid probe was prepared[84] from intermediate **16** (Figure 9). Protecting groups were selectively repositioned (**17**, **18**) and the required phosphate esters were installed (**19**) to give the P-2-linked Ins(1,2,4,5)P_4 (**20**).

Since our earlier work on tethered IP_3 had yielded racemic material, a new route (Figure 10) was developed to D-*myo*-P-1-*O*-(aminopropyl)-Ins(1,4,5)P_3 affinity probes based on the scheme in Figure 9.[85] Thus, manipulation of acetal **21** via enol acetate **22** and protection of C-3 as a benzyl ether led to rearranged intermediate **23**. The chiral IP_3 derivative **24** was converted with [^{3}H]BZDC-NHS ester to photoprobe **25**, which has been employed to photoaffinity label the PIP_2-binding pleckstrin homology (PH) domain of phospholipase C-δ_1.[86]

Onward to IP_6 Derivatives

Our original synthesis of *meso*-P-2-*O*-(aminohexyl)-Ins(1,2,3,4,5,6)P_6 is shown in Figure 11.[87a] Ketal **26** was converted to key intermediate **27**, the linker was

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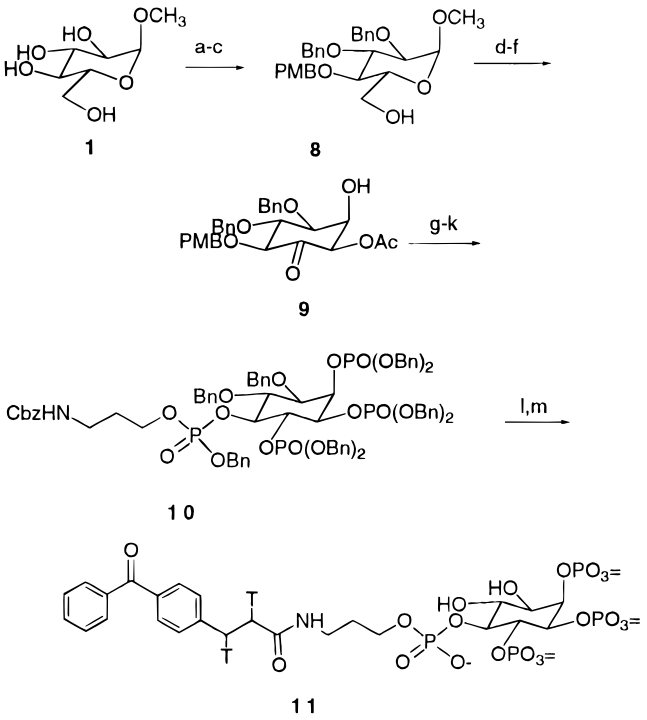

Figure 7. Synthesis of P-5-linked Ins(1,2,5,6)P_4. Reagents: (a) *p*-methoxybenzaldehyde dimethyl acetal, pTsOH, DMF; (b) BnBr, NaH, DMF; (c) TMSCl, $NaBH_3CN$; (d)−(f) "Ferrier sequence", steps (d)−(f) in Figure 5; (g) $NaBH(OAc)_3$, AcOH; (h) NaOH, MeOH; (i) "phosphorylation", steps (m) and (n) in Figure 5; (j) $(NH_4)_2Ce(NO_3)_6$; (k) "linker attachment", steps (j) and (k) in Figure 5; (l) "deprotection sequence", steps (o) and (p) in Figure 5; (m) [^{3}H]BZDC-NHS, DMF, 0.25 M TEAB, pH 8.3; then DEAE-cellulose (HCO_3^- form).

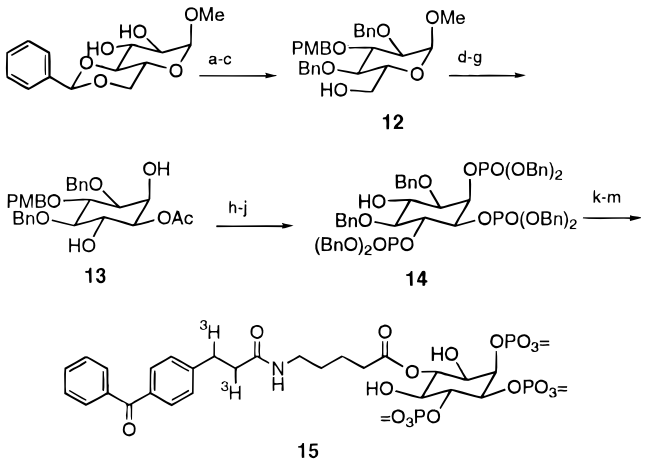

Figure 8. Synthesis of 4-acylated D-*myo*-Ins(1,2,6)P_3. Reagents: (a) $Bu_4N^+HSO_4^-$BnBr, CH_2Cl_2, aqueous NaOH; (b) PMB-Cl, NaH, DMF; (c) DIBAL, CH_2Cl_2; (d)−(g) Ferrier sequence; (h) NaOH, MeOH; (i) phosphorylation; (j) $(NH_4)_2Ce(NO_3)_6$, MeCN−H_2O (9:1); (k) $CbzNH(CH_2)_4COOH$, DCC, DMAP; (l) deprotection sequence; (m) "photolabel attachment", step (m) in Figure 7.

attached (**28**), and protecting groups were cleaved (**29**). The IP_6 affinity column **30b** allowed purification of Ins(1,3,4)P_3 5/6-kinase[23] and IP_6 kinase,[88] and the [^{3}H]-BZDC-IP_6 probe **30a** has proven to be a powerful tool for labeling the active sites of proteins important in intracellular protein trafficking (J. D. Olszewski, A. Chaudhary, B. Mehrotra, unpublished results). For this material, the asymmetric route from glucose made

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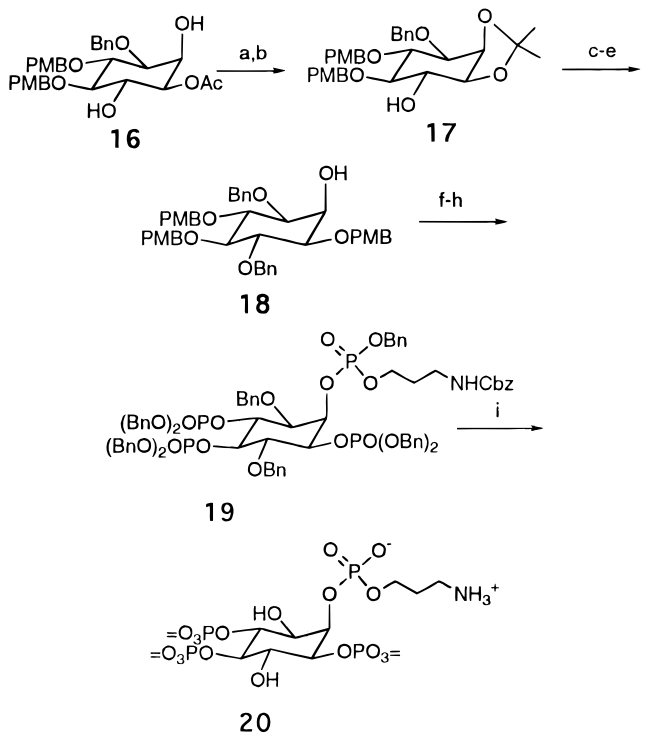

Figure 9. Synthesis of D-*myo*-P-2-*O*-(aminopropyl)-Ins(1,2,4,5)-P_4. Reagents: (a) NaOH, MeOH; (b) TsOH, 2,2-dimethoxypropane, Me_2CO; (c) NaH, BnBr, DMF; (d) TsOH, Me_2CO−H_2O; (e) Bu_2SnO, *n*-Bu_4NI, toluene; then PMB-Cl; (f) linker attachment; (g) $(NH_4)_2Ce(NO_3)_6$, MeCN−H_2O; (h) phosphorylation; (i) deprotection sequence.

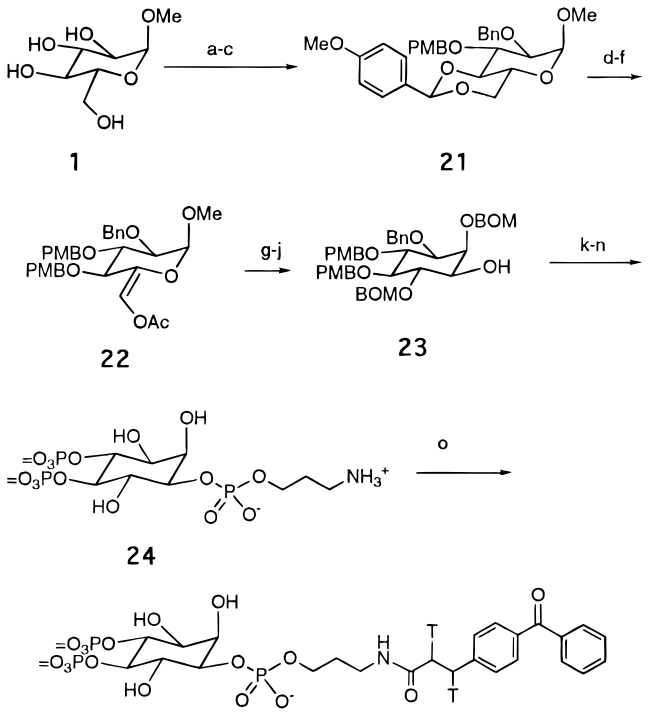

Figure 10. Synthesis of D-*myo*-P-1-*O*-(aminopropyl)-Ins(1,4,5)-P_3. Reagents: (a) *p*-methoxybenzaldehyde dimethyl acetal, pTsOH, DMF; (b) BnBr, CH_2Cl_2, nBu_4NHSO_4, 10% aqueous NaOH; (c) NaH, PMB-Cl, DMF; (d) DIBAL-H, CH_2Cl_2; (e)−(h) Ferrier sequence; (i) BOM-Cl, Bu_4NBr, H^+ sponge, MeCN; (j) NaOH, MeOH; (k) linker attachment; (l) DDQ, wet CH_2Cl_2; (m) phosphorylation; (n) deprotection; (o) photolabel attachment.

little sense. On the other hand, since receptors could have chiral recognition sites, a P-1-tethered IP_6 (Figure 11, bottom) was initially prepared in racemic

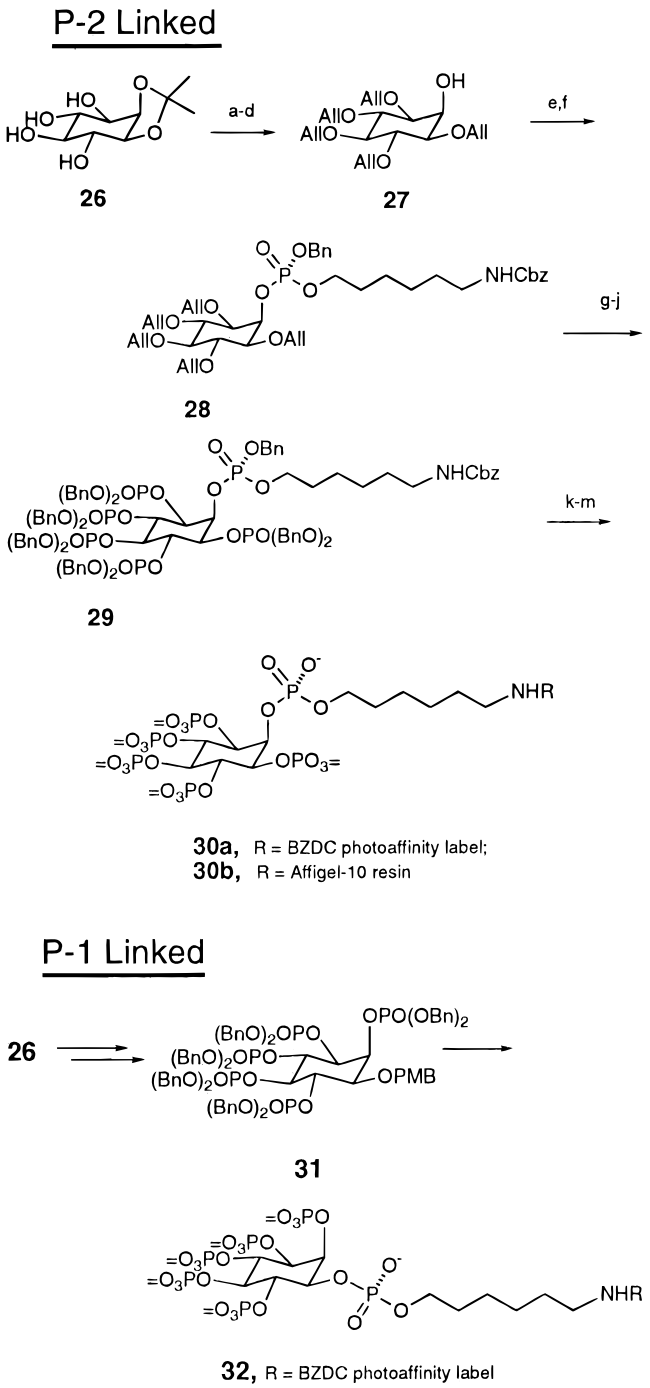

Figure 11. Synthesis of *meso*-P-2-*O*-(aminohexyl)-Ins(1,2,3,4,5,6)-P_6. Reagents: (a) NaH, AllBr, DMF; (b) H_3O^+, CH_3OH; (c) Bu_2-SnO; (d) AllBr, DMF; (e)−(f) linker attachment; (g) $(Ph_3P)_3RhCl$, C_2H_5OH; (h) H_3O^+, CH_3OH; (i) phosphorylation; (j) *m*-CPBA; (k)−(l) deprotection; (m) BZDC-NHS, TEAB, pH 8.5 for R = BZDC; Affigel-10, $NaHCO_3$ buffer for R = Affigel-10 resin.

form[87b] by conversion of **26** to protected intermediate **31**. [^{3}H]BZDC-P-1-(aminohexyl)-linked IP_6 **32** has shown different (and mostly lower) affinities for several IP_6 binding proteins (coatomer, AP-2, synaptotagmin, and cytosolic IP_6BP) relative to **30a** (A. Chaudhary, A. A. Profit, J. Chen, unpublished results). Preparation of the chiral derivative via a Ferrier route awaits justification from biological data.

Phosphoinositide Polyphosphates

Lipid-Modified Analogs of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. A number of syntheses of phosphoinositide polyphosphates have recently been presented.[89−92] However, none of the published routes allowed access to tritium-labeled materials or permitted site-specific incorporation of a reporter group. Thus, we followed two strategies for introducing reporter groups: modification of the lipid moiety and insertion of a linker on the phosphodiester phosphate. Figure 12 illustrates the structures of several common phospholipids and a variety of fluorophore-linked and photophore-linked PIP_2-type probes.

The acyl-modification strategy is described first. Figure 13 compares the chemical structures of the P-1-tethered photoaffinity labels used as probes for Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding sites with both the native phosphatidylinositol polyphosphates PtdIns-(4,5)P_2 (PIP_2) and PtdIns(3,4,5)P_3 (PIP_3) and with the target acyl-modified PIP_2 and PIP_3 photoaffinity labels. Lipid-modified analogs of PIP_2 and PIP_3 were prepared in which either a 6-aminohexanoyl group or a 12-aminododecanoyl group was installed in the *sn*-1 position.[93] This strategy allowed attachment of a reporter group (e.g., BZDC photophore, NBD fluorophore) that could either reside in the lipid bilayer or interact with lipid-binding sites on a macromolecular target.

The synthesis (Figure 14) followed a convergent approach, beginning with selective *sn*-1-*O*-acylation[94] of a 3-PMB-protected chiral glycerol synthon, followed by acylation of the *sn*-2 position and oxidative deprotection with DDQ to give the desired 1,2-*O*-diacylglycerol derivative. Reaction with benzyl(*N*,*N*-diisopropylamino)chlorophosphine afforded rather labile phosphoramidites **33**, which were then condensed with the appropriately protected D-*myo*-inositol derivative. Intermediate **23**[85] from the P-1-tethered Ins(1,4,5)P_3 route was used in making PIP_2 analogs, while intermediate **5**[65] from the P-1-tethered Ins(1,3,4,5)P_4 route was used for making PIP_3 analogs. In these intermediates (e.g., **23**), benzyl (Bn) or benzyloxymethyl (BOM) groups protected final hydroxyl groups and PMB groups masked future phosphomonoesters. Deprotection of the PMB groups, phosphorylation, and finally hydrogenolysis and ion exchange chromatography afforded the aminoacyl-tethered analogs of PIP_2 and PIP_3. Attachment of the photophore using [^{3}H]-BZDC-NHS ester[95] gave the desired photolabile probes **35**. In addition, we prepared an extremely high specific activity, nonhydrolyzable ether analog of PIP_2 (**36**) for binding assays (J. Chen, unpublished results). Thus, condensation of a 1,2-*O*-di-10-undecenyl-*sn*-glycerol-derived phosphoramidite with the IP_3 intermediate **24** led to a protected diether PIP_2 analog. Hydrogenolysis with carrier-free tritium (1 atm) followed by hydrogen (4 atm) gave the tritium-labeled diether **36** with >200 Ci/mmol specific activity.

Triester Analogs of PIP_2 and PIP_3. A novel concept for photolabeling of PIP_2 and PIP_3 binding proteins was conceived by Dr. Q.-M. Gu in which the reporter group could be positioned at the water/

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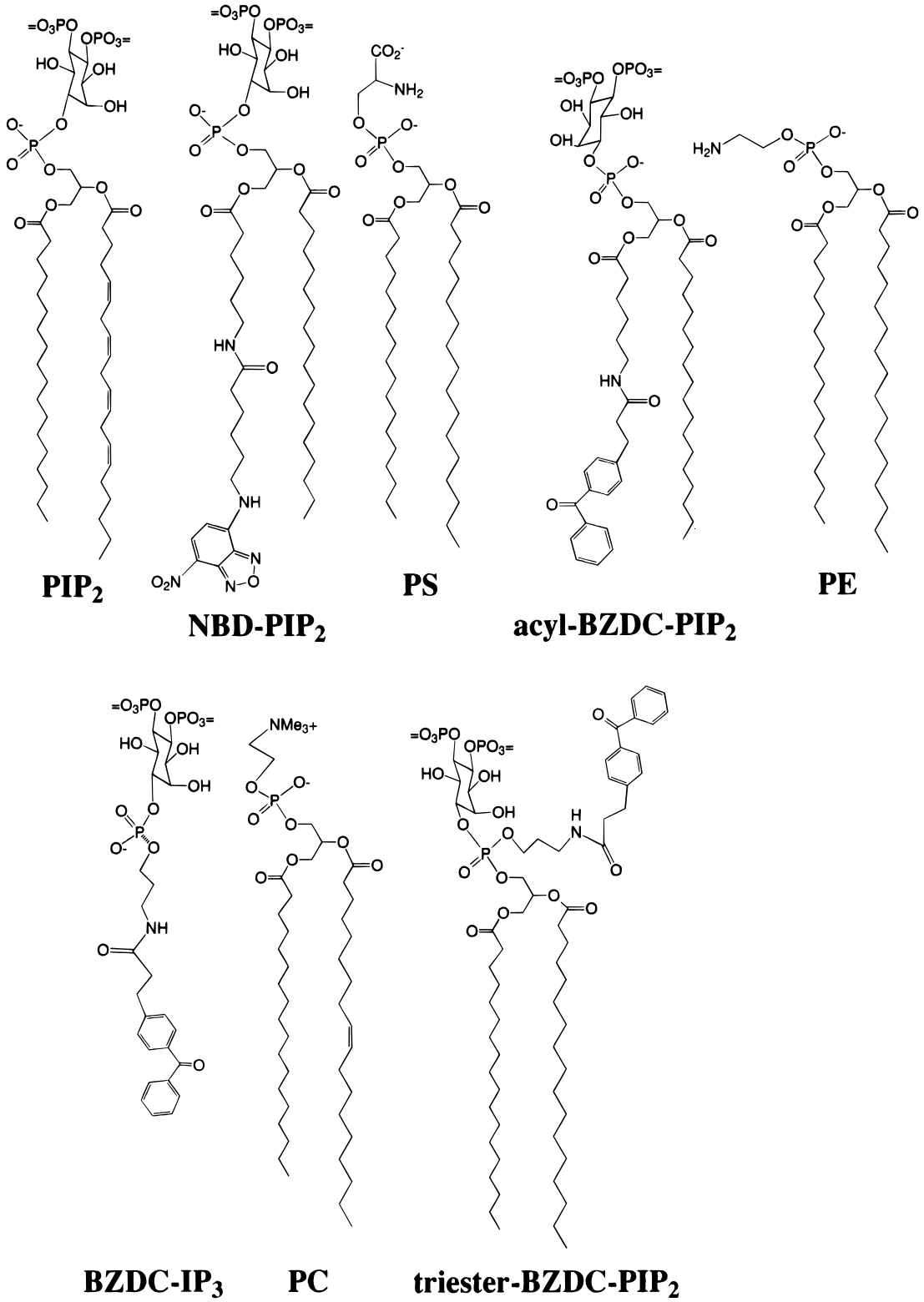

Figure 12. Naturally-occuring phospholipids (PIP_2, PtdIns(4,5)P_2; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine) and selected PIP_2 analogs with photophoric (BZDC) or fluorophoric (NBD) reporter groups.

phospholipid head group interface.[96] The PIP_2 analog containing this modification is depicted in Figure 12 at the bottom right. In this strategy, both the diacylglycerol bilayer anchor and the 4,5-bisphosphate (or 3,4,5-trisphosphate) recognition groups were retained. The synthetic route (Figure 15) to the triesters hinges on the use of the 2-cyanoethyl protecting group for the inosityl P-1 phosphate that also forms the phosphodiester linkage to the diacylglycerol moiety.

(96) Gu, Q.-M.; Prestwich, G. D. *J. Org. Chem.*, in press.

The phosphatidylinositol derivatives were assembled convergently. For the PIP_3 analog, dipalmitoylglycerol was converted to the 2-cyanoethyl *N,N*-diisopropylphosphoramidite derivative and coupled to the inositol **5**[65] to give the adduct **37a**. The 2-cyanoethyl phosphotriester was stable during the subsequent deprotection and phosphorylation steps, and the phosphorylated product **38a** was converted to the 3-aminopropyl-tethered phosphotriester **39a** during hydrogenolysis. The aminopropyl group of **39a** was readily converted to the [^{3}H]BZDC-PIP_3 derivative **40a**. The

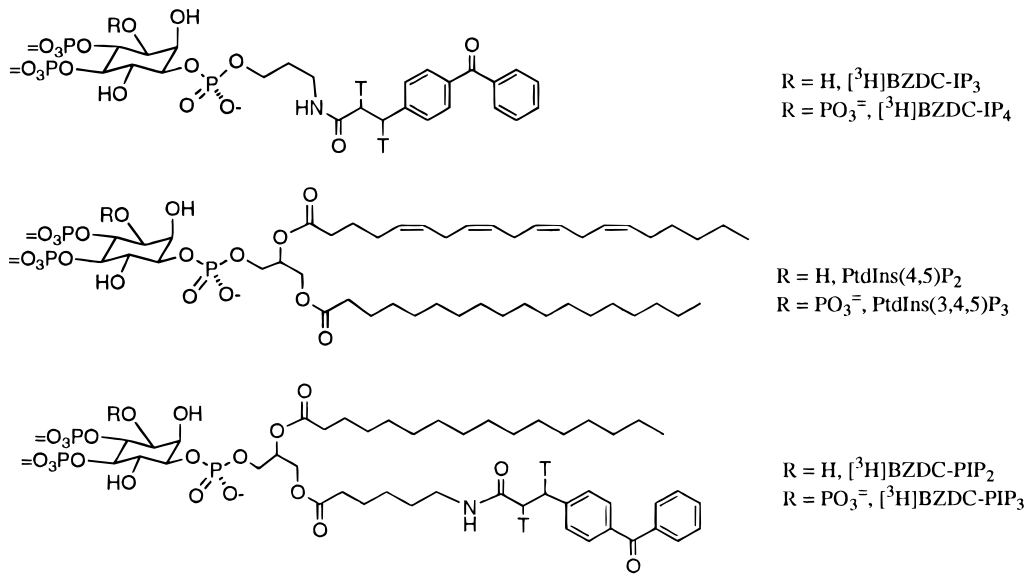

Figure 13. Comparison of P-1-tethered photoaffinity probes for IP_3 and IP_4 with PIP_2, PIP_3, and acyl-modified derived photoaffinity analogs of PIP_2 and PIP_3.

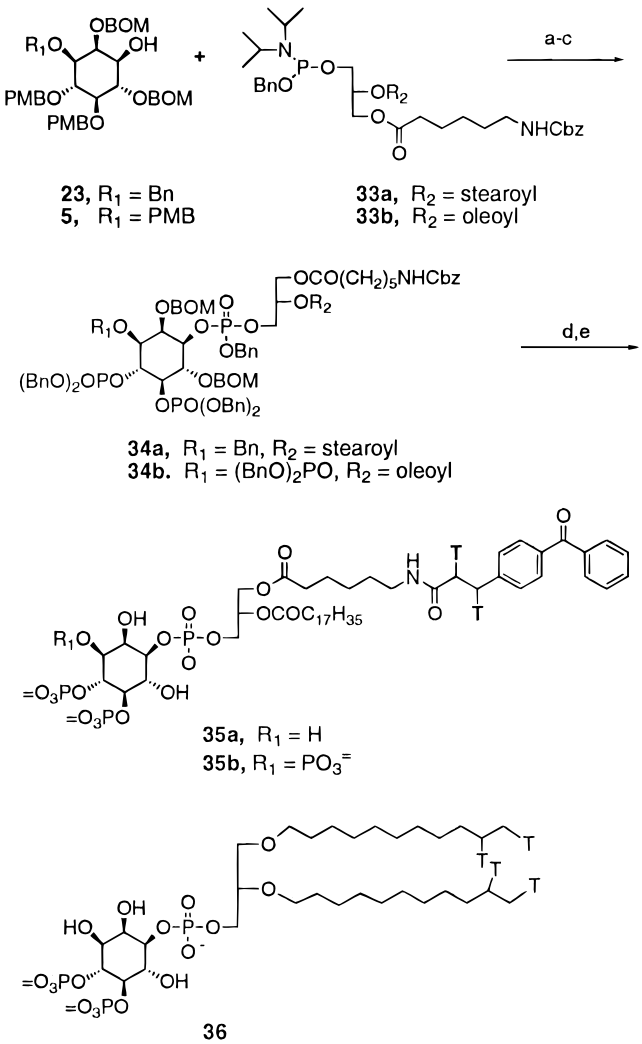

Figure 14. Synthesis of acyl-modified PIP_2 and PIP_3 analogs. Reagents: (a) 1-*H*-tetrazole, *m*-CPBA; (b) DDQ, CH_2Cl_2; (c) phosphorylation; (d) deprotection in *t*BuOH−H_2O, $NaHCO_3$; (e) photolabel attachment.

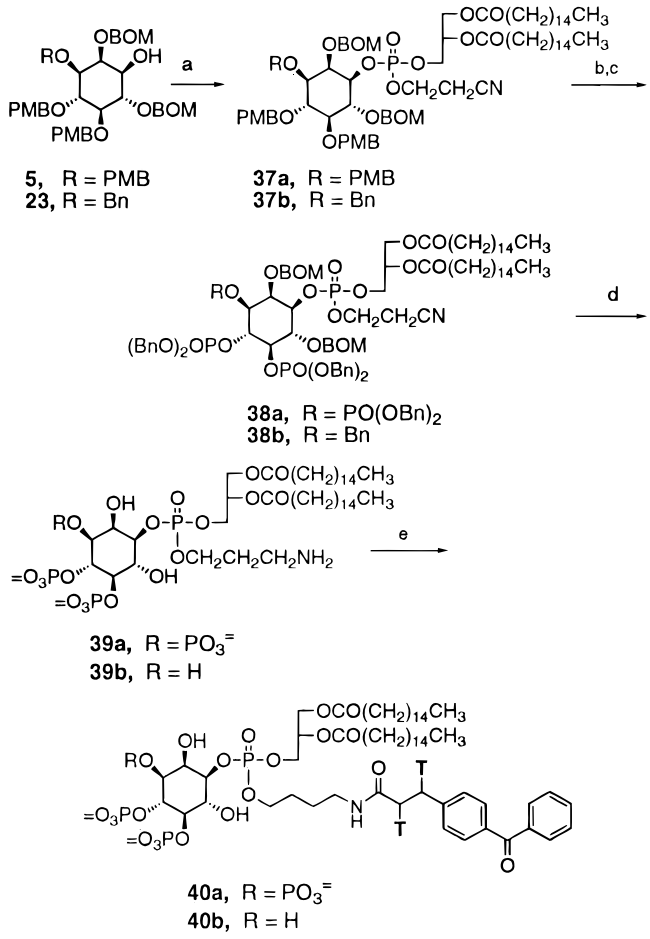

Figure 15. Synthesis of triester analogs of PIP_2 and PIP_3. Reagents: (a) diacylglyceryl cyanoethyl phosphoramidite, tetrazole, CH_2Cl_2; then *m*-CPBA; (b) DDQ, CH_2Cl_2; (c) phosphorylation; (d) deprotection (and reduction of nitrile) in *t*BuOH−H_2O, $NaHCO_3$; (e) photolabel attachment.

PIP_2 analog **40b** was prepared following an analogous route, but using the key intermediate **23**[85] as the inositol component.

The 2-cyanoethyl group provided three advantages. First, the high reactivity of the 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite ensured that a bulky primary alcohol and a sterically-congested secondary alcohol could both form efficient new O−P bonds. Second, the 2-cyanoethyl phosphite derivatives were more stable to chromatography than the corresponding benzyl phosphites used for the PIP_2 and PIP_3 phosphodiester route above. Third, not only could the

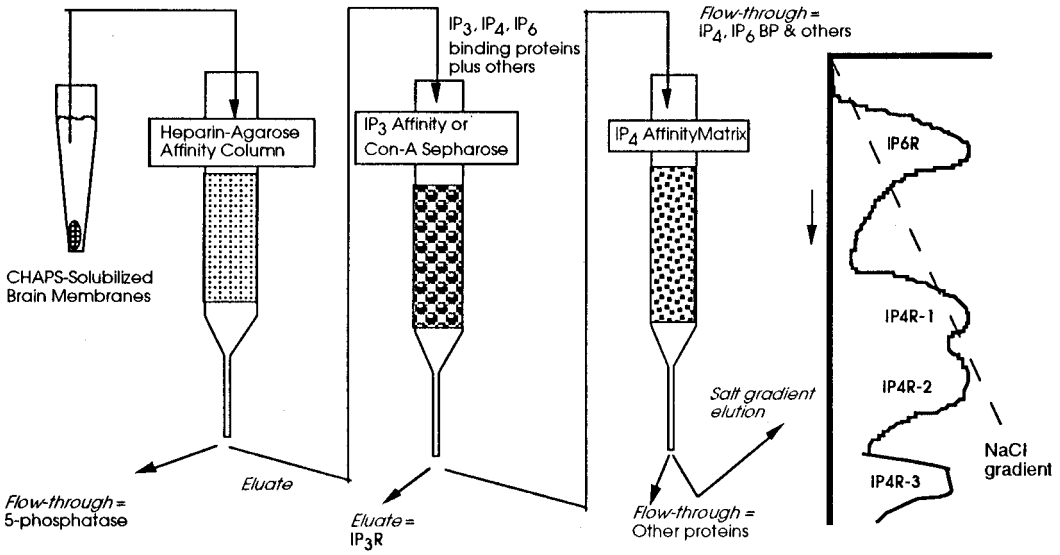

Figure 16. Application of IP_4 affinity resin to purification of IP_4, IP_6, and PIP_3 binding proteins. The first eluted fraction IP_6R was identified as AP-2; IP_4R-1 and IP_4R-2 are still unidentified; IP_4R-3 has recently been described as the PIP_3 binding protein centaurin.

2-cyanoethyl group serve as the source of the 3-aminopropyl linker, but it could be removed by β-elimination using a trialkylamine to provide high yields of the phosphodiesters PIP_2 and PIP_3 (see Figure 13).

Protein Purification and Active Site Labeling

The synthesis of the probes was only the beginning. We have, Pandora-like, succumbed to the temptation to use our newly made keys to try to unlock the black boxes of cellular signaling and intracellular protein transport. Fortunately, these opened boxes have produced not pestilence but treasures beyond our expectations. Selected recent examples are highlighted below.

Labeling with [^{3}H]BZDC-Ins(1,4,5)P_3. Our first results with purification and labeling the IP_3R using P-1-*O*-(3-aminopropyl)-linked derivatives[39] mapped the IP_3 binding site. More recently, we showed that PIP_2 binding proteins could also be studied with BZDC-IP_3.[86] Thus, the δ_1-isozyme of PLC contains a PH domain responsible for binding of PLC to PIP_2-rich lipid bilayers;[86b] catalysis of phosphodiester hydrolysis takes place at a different site. Two PLC-β isozymes lack the PH domain, yet still hydrolyze PIP_2 to IP_3 and DAG. Photoaffinity labeling of recombinant proteins comprising either the full-length PLC-δ_1 or only the PH domain with [^{3}H]BZDC-IP_3 modified the N-terminal base-rich sequence unique to this isozyme.[86] The two PLC-β isozymes were not labeled by this probe. This is the first evidence for an important hydrophobic interaction site of a PH domain in addition to the 4,5-bisphosphate recognition role.

Purification on an IP_4 Affinity Column and Labeling with [^{3}H]BZDC-Ins(1,3,4,5)P_4. Purification by IP_4 affinity chromatography and photoaffinity labeling of putative IP_4 binding proteins from rat brain[27,77] led to the characterization of four proteins (Figure 16). The first eluting heterooligomer had the highest affinity for IP_6, and was identified[59] as assembly protein AP-2, an essential component in the formation of clathrin-coated pits during endocytosis. The second and third eluting proteins are heterooligomers with as yet undescribed functions. The fourth protein eluted at much higher ionic strength from this IP_4 affinity column, and the protein was very efficiently labeled with [^{3}H]BZDC-IP_4. This protein has been cloned and its cDNA sequenced, revealing an unexpected chimeric structure that includes (in addition to the IP_4 binding region) a protein kinase C zinc finger, and several domains suggesting interactions with actin and other cytoskeletal proteins. This protein, named centaurin, is the first protein clearly demonstrated to show preferential binding to PIP_3 relative to IP_4 or any other PI pathway metabolite.[97]

Photolabeling with [^{3}H]BZDC-Ins(1,2,3,4,5,6)-P_6 and Purifications with an IP_6 Affinity Column. P-2-Linked [^{3}H]BZDC-IP_6 was recently prepared[98] and used along with P-1-linked [^{3}H]BZDC-IP_4 in labeling synaptotagmin C2B domains,[57] Golgi coatomer proteins,[60] liver cytosolic IP_6 binding proteins,[61] and assembly proteins AP-2 and AP-3.[58,69] The P-2-linked IP_6 resin has also proven to be valuable for enzyme purification. For example, an Ins(1,3,4) 5/6-kinase was purified 20-fold in a final step to give homogeneous protein.[23] More recently, the IP_6 kinase responsible for biosynthesis of IP_7 from IP_6 and ATP was purified.[88]

Studies with Modified PIP_n. The PIP_2 and PIP_3 probes have just been completed.[93,96] Early studies with binding of BZDC-IP_3 and the triester and acyl-modified PIP_2 photolabels to profilin show strikingly different results for each photoprobe. A fluorescent probe, NBD-PIP_2 (see Figure 12), was synthesized[93] for studies[99] on the interaction of a highly basic peptide with the acidic phospholipids PIP_2 and phosphatidylserine (PS) in bilayers. The basic peptides caused sequestration of PIP_2 and PS into lateral domains, thus preventing hydrolysis of PIP_2 by PLC.[99] The inhibition was released upon phosphorylation of serines in the basic peptides, thereby ablating the necessary electrostatic interaction. Selective labeling of PIP_3 binding proteins with the triester will be reported elsewhere.

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Conclusions

Phosphoinositide polyphosphate and inositol polyphosphate recognition sites in target proteins are rich in basic arginine and lysine residues that are protonated at physiological pH. The binding site for a given arrangement of two or more equatorial, anionic phosphates is determined by the number and 3-dimensional display of these bases. Over the past six years, we have sought to understand these binding sites by "touching all the bases" with PIP_n and IP_n substrates bearing reporter groups. A molecular level understanding of regulation of cellular signaling and protein trafficking can be pursued with the palette of phosphoinositide pathway photoprobes described in this Account.

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