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The Synthesis of Membrane Permeant Derivatives of *myo*-Inositol 1,4,5-Trisphosphate

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In order to enable the study of the intracellular second messenger D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) and its receptors ($InsP_3Rs$), it has been desirable to develop protected derivatives of $InsP_3$ that are able to enter the cell, upon extracellular application. The subsequent removal of the lipophilic protecting groups, by intracellular enzymes, releases $InsP_3$ and leads to the activation of $InsP_3Rs$. Two syntheses of D-*myo*-inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester (D-$InsP_3$/BM) and one of L-$InsP_3$/BM are reported. It is demonstrated that extracellular application of the D-enantiomer results in Ca^{2+} release, which is thought to occur via $InsP_3Rs$. Application of the L-enantiomer resulted in little Ca^{2+} release.

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Introduction

The intracellular study of phosphorylated small molecules is complicated by the inability of these compounds to passively diffuse across the cell membrane, a characteristic that is conferred by their overall negative charge. However, the delivery of multiply charged compounds, across the lipophilic cell membrane, to an intracellular location can be achieved by the masking of the charged moieties with lipophilic groups.[1] To be useful, these groups must allow the compound to diffuse across the cell membrane before the lipophilic groups are removed by intracellular enzymes to reveal the biologically active compound. Early work on membrane permeant phosphate derivatives focused on the synthesis of simple phosphate triesters.[1,2] However, it was found that the intracellular half-lives of these compounds are very long, ranging from minutes or hours to days. The intracellular stability of these groups results from the lack of specific enzymes to hydrolyze them and hence their intracellular cleavage is reliant on uncatalyzed chemical hydrolysis rather than enzymatic hydrolysis.[1]

Lipophilic protecting groups that are stable outside of the cell, but cleaved efficiently by intracellular enzymes, have been used to furnish compounds that are of more use to biological investigators. One such group is the acetoxymethyl (AM) ester. This protecting group was first employed to mask the carboxylate group of penicillins and hence improve their bioavailability, with the AM group removed by esterase enzymes once the drug had reached its target.[1,3] The AM group shows improved lability within cells (compared to the simple phosphate triesters) as the methylene linker removes the ester moiety from the steric bulk of the protected compound, allowing non-specific esterase enzymes to interact with it. AM groups have also been used to mask phosphate groups, the esterase enzymes hydrolyze the AM ester and the spontaneous removal of formaldehyde reveals the free phosphate (Fig. 1).

Tsien and co-workers have applied this technology to the synthesis of membrane permeant analogues of the intracellular second messenger D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$).[4] The use of AM, propionyloxymethyl (PM), and butyryloxymethyl (BM) moieties to protect the phosphate groups of racemic $InsP_3$ was reported (Fig. 2).[4] The masked $InsP_3$ diffused through the cell membrane and on entering the cell, the protecting groups were removed by esterase enzymes to give formaldehyde and $InsP_3$, which was demonstrated to evoke release of intracellular Ca^{2+}, presumably via activation of $InsP_3$ receptors ($InsP_3Rs$).[4] Tsien has also reported a caged and membrane permeant analogue of $InsP_3$.[5] Schultz and co-workers have applied this

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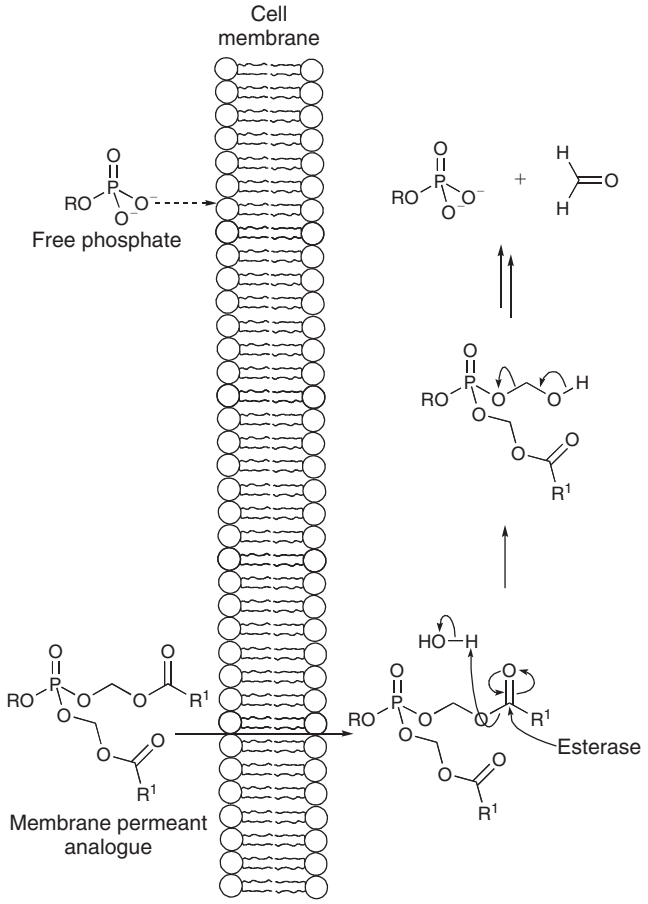


Fig. 1. Acetoxymethyl-protected phosphates and derivatives can passively diffuse across the cell membrane. Hydrolysis by intracellular esterase enzymes reveals the biologically active compound and formaldehyde. $R^1 = CH_3$ (AM), CH_2CH_3 (PM), $(CH_2)_2CH_3$ (BM).

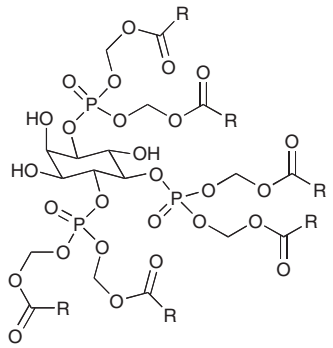


Fig. 2. The membrane permeant analogues of *rac*-$InsP_3$ reported by Tsien and co-workers: *rac-myo*-inositol 1,4,5-trisphosphate hexakis(acetoxymethyl) ester ($InsP_3$/AM, **1**); *rac-myo*-inositol 1,4,5-trisphosphate hexakis(propionyloxymethyl) ester ($InsP_3$/PM, **2**); *rac-myo*-inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester ($InsP_3$/BM, **3**).[4]

approach to the synthesis of a membrane permeant derivative of *myo*-inositol-1,3,4-trisphosphate,[6] membrane permeant phosphatidylinositol polyphosphates,[7] and *myo*-inositol 3,4,5,6-tetrakisphosphate.[8]

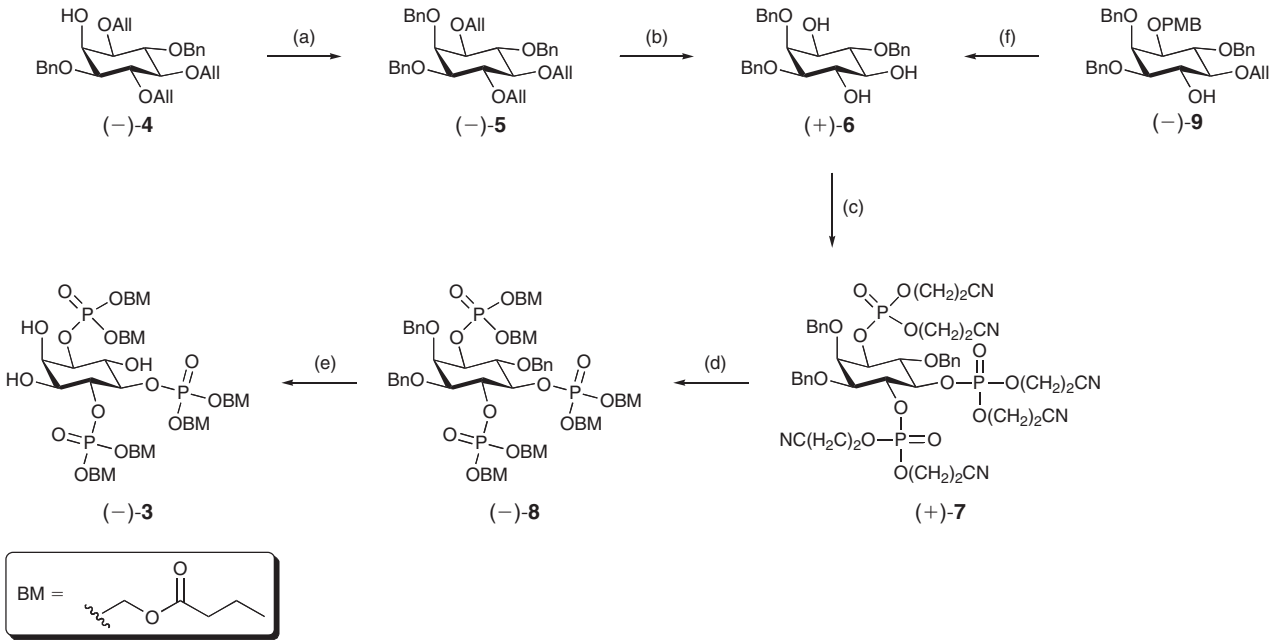
The racemic butyryloxymethyl ester of $InsP_3$ ($InsP_3$/BM, **3**)[4] and the caged membrane permeant $InsP_3$ analogue[5] have proved to be of great use to those studying the involvement of $InsP_3$ in intracellular signalling.[5,9–13] This led us to develop syntheses of both the D- and the L-$InsP_3$/BM for use as chemical probes of $InsP_3$ signalling, based on our previous investigations into the synthesis of the phosphatidylinositol polyphosphates.[14–20] D-$InsP_3$/BM has proved to be invaluable in the study of $InsP_3$ and Ca^{2+} signalling[12,13,21–27] and therefore we report the synthesis of both enantiomers of $InsP_3$/BM (**3**) and demonstrate the differing Ca^{2+}-releasing properties of each compound.

Results and Discussion

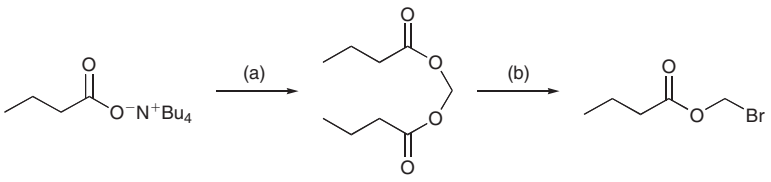
Synthesis of Compounds

In the first approach to the synthesis of D-$InsP_3$/BM, (−)-**3** (Scheme 1), the route utilized by Ozaki et al.[28] in the synthesis of D-$InsP_3$ was employed to furnish the optically pure alcohol (−)-**4** {$[\alpha]_D^{20} = -14.6$ (*c* 0.5, $CHCl_3$) [lit.,[28] $[\alpha]_D^{16} = -14.3$ ($CHCl_3$)]}. Benzylation of the axial alcohol followed by Wilkinson's catalyst [$Rh(PPh_3)_3Cl$]-mediated isomerization of the allyl groups and subsequent methanolysis of the resulting enol ether afforded the known triol (+)-**6** {$[\alpha]_D^{20} = +12.3$ (*c* 0.40, $CHCl_3$) [lit.,[29] $[\alpha]_D = +12.4$ (*c* 0.80, $CHCl_3$)]} in good yield (81%).[28–30] 1*H*-Tetrazole-catalyzed phosphitylation of the triol (+)-**6** with *N*,*N*-diisopropylamino bis-(2-cyanoethoxy)phosphine, followed by P(III) to P(V) oxidation using *m*CPBA, afforded the fully protected $InsP_3$ analogue (+)-**7** (δ_P −2.5, −2.5, and −2.7 ppm in $CDCl_3$) in 86% yield. Treatment of the cyanoethyl moieties with triethylamine furnished the presumed triethylammonium salt of $InsP_3$, which reacted with bromomethylbutyrate (**12**) to yield the fully benzylated $InsP_3$/BM (−)-**8** (δ_P −4.6, −4.2, and −3.9 ppm). This reaction was found to be capricious and only proceeded in poor to moderate yield (29–55%). The poor yields for this reaction reflects the fact that six eliminations and six substitutions have to occur in order to furnish the desired product, and that the triethylammonium salt of the phosphate group is likely to be a poor nucleophile. Hydrogenolysis of the benzylated $InsP_3$/BM (−)-**8** using palladium acetate and palladium trifluoroacetate as catalysts afforded $InsP_3$/BM (−)-**3** (δ_P −4.1, −3.8, and −3.6 ppm in MeOD) in good yield (88%). Palladium acetate and palladium trifluoroacetate were employed as Tsien has previously demonstrated that this reaction fails when conducted with Pd on carbon as a catalyst.[4] The use of neat acetic acid as the solvent during hydrogenolysis was intended to maintain the product in a fully protonated state and therefore minimize possible phosphate group migrations. $InsP_3$/BM (−)-**3** was observed to be fairly unstable and broke down if left at room temperature or in solvent for more than a few hours.

The L-isomer (+)-**3** was synthesized in an analogous manner using the optically antipodal alcohol (+)-**4** {$[\alpha]_D^{20} = +14.6$ (*c* 0.5, $CHCl_3$) [lit.,[28] $[\alpha]_D^{16} = +14.2$ ($CHCl_3$)]}, which was also furnished during the menthoxyacetyl-based diastereomeric resolution. The yields obtained, shown in



Scheme 1. Synthesis of D-$InsP_3$/BM (−)-**3**. Yields in parentheses refer to the L-isomer. Reagents and conditions: (a) NaH, BnBr, DMF, 0°C → RT, 81% (85%). (b) (i) Wilkinson's catalyst [$Rh(PPh_3)_3Cl$], Hünig's base, EtOH, reflux; (ii) AcCl, MeOH, CH_2Cl_2, 75% (80%). (c) (i) *N*,*N*-Diisopropylamino bis-(2-cyanoethoxy)phosphine, 1*H*-tetrazole, CH_2Cl_2; (ii) *m*CPBA, 86–93% (64%). (d) Et_3N, CH_2Cl_2 then bromomethylbutyrate and Hünig's base, 29–48% (55%). (e) $Pd(CF_3CO_2)_2$, $Pd(CH_3CO_2)_2$, AcOH, H_2 (1 atm), 15°C, 88–91% (88%). (f) (i) Wilkinson's catalyst [$Rh(PPh_3)_3Cl$], Hünig's base, EtOH, reflux; (ii) conc. HCl, MeOH, reflux, 66% yield over two steps.



Scheme 2. Synthesis of bromomethylbutyrate (**12**). Reagents and conditions: (a) (i) 2 M NaOH solution, tetrabutylammonium hydrogen sulfate, 56%; (ii) CH_2Cl_2 reflux, 2 days. (b) TMSBr, RT, 24 h, 48%.

parentheses (Scheme 1), were generally comparable with those seen for the enantiomeric material.

The second synthetic route (Scheme 1) is based on that employed in the previously reported synthesis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2].[19] The synthesis of D-$InsP_3$/BM (−)-**3** commenced from the optically pure alcohol (−)-**9** {$[\alpha]_D^{25} = -1.8$ (*c* 1.6, $CHCl_3$) [lit.,[19] $[\alpha]_D^{20} = -0.6$ (*c* 0.6, $CHCl_3$)]}, which was obtained from *myo*-inositol orthoformate, as previously reported.[16,19] Wilkinson's catalyst-mediated isomerization of the allyl group to the corresponding enol ether, followed by acid catalyzed methanolysis of the enol ether and the 1-position PMB group, afforded the known triol (+)-**6** {$[\alpha]_D^{20} = +12.1$ (*c* 0.88, $CHCl_3$) [lit.,[29] $[\alpha]_D = +12.4$ (*c* 0.80, $CHCl_3$)]}.[28–30] 1*H*-Tetrazole-catalyzed phosphitylation of the triol (+)-**6** with *N*,*N*-diisopropylamino bis-(2-cyanoethoxy)phosphine, followed by P(III) to P(V) oxidation using *m*CPBA, afforded the fully protected $InsP_3$ analogue (+)-**7** [δ_P −2.5 and −2.7 ppm (2:1) in $CDCl_3$]. Treatment of the cyanoethyl moieties with triethylamine furnished the presumed triethylammonium salt of $InsP_3$, which reacted with bromomethylbutyrate (**12**) to yield the fully benzylated $InsP_3$/BM (−)-**8** (δ_P −4.7, −4.3, and −4.0 ppm in $CDCl_3$). Hydrogenolysis of the benzylated $InsP_3$/BM (−)-**8** using palladium acetate and palladium trifluoroacetate as catalysts afforded $InsP_3$/BM (−)-**3** (δ_P −4.1, −3.8, and −3.6 ppm in MeOD).

Methylene dibutyrate (**11**) was furnished heating the tetrabutylammonium salt of butyric acid (**10**) in dichloromethane under reflux, as described by Holmberg and Hansen (Scheme 2).[31] The bromomethyl butyrate (**12**) was synthesized as described by Tsien and co-workers by treating methylene dibutyrate (**11**) with trimethylsilyl bromide to yield the desired compound (**12**).[4,32] The compound was purified by Kugelrohr distillation before each use, to ensure optimum yield in the subsequent reaction.

Biological Evaluation of Compounds

Berridge and Irvine were the first to demonstrate that D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) release causes an increase in intracellular Ca^{2+} concentration.[33] $InsP_3$ is a key second

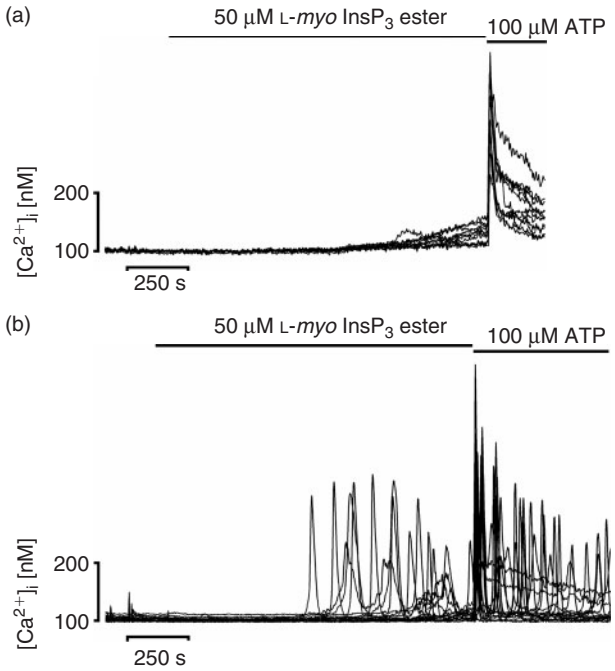


Fig. 3. D-$InsP_3$/BM ester (−)-**3**, but not L-$InsP_3$/BM ester mobilizes calcium stores. Fura-2-loaded human embryonic kidney (HEK) cells were incubated with the forms of $InsP_3$ ester shown in the figures. Adenosine triphosphate stimulates the production of $InsP_3$ in HEK cells, and was used in these experiments as a positive control. Please note that the calcium signals observed during incubation with D-$InsP_3$/BM (−)-**3** reflected release of the ion from intracellular stores, since the compound was applied in calcium-free medium. The traces in (a) and (b) are from single experiments, and are representative of several trials. The trace in (b) shows Ca^{2+} release from multiple cells on the same coverslip and gives a representative picture of the effect of D-$InsP_3$/BM (−)-**3** on Ca^{2+} release.

messenger that is released as a result of cell-surface receptor activation, triggering the phospholipase C (PLC)-mediated hydrolysis of PtdIns(4,5)P_2 to form diacylglycerol (DAG) and $InsP_3$. The lipophilic DAG remains in the plane of the cell membrane and effects signal transduction by activation of protein kinase C (PKC). $InsP_3$, which is hydrophilic, diffuses into the cytosol, and activates $InsP_3$Rs to release Ca^{2+}. Ca^{2+} is an almost universal intracellular messenger, controlling a diverse range of cellular processes, such as gene transcription, muscle contraction, and cell proliferation.[34–36] The importance of Ca^{2+} signalling has led to prolific investigation in this field, however, there are very few membrane permeant small molecule $InsP_3$R agonists or antagonists.

To investigate the Ca^{2+}-releasing ability of D- and L-$InsP_3$/BM Fura-2-loaded human embryonic kidney (HEK) cells were incubated with either D- or L-$InsP_3$/BM (Fig. 3). L-$InsP_3$/BM (+)-**3**, which is ~1000-fold less potent at $InsP_3$Rs than D-$InsP_3$/BM (−)-**3**,[37] showed little evidence of Ca^{2+}-releasing ability (Fig. 3a). D-$InsP_3$/BM (−)-**3**, however, demonstrated Ca^{2+}-releasing ability after an initial incubation period (Fig. 3b), which is presumably related to the deprotection of the BM esters. This time delay is to be expected, as all six esters have to be removed before the compound will induce $InsP_3$R activity. It should be noted that cells that *did not* respond to L-$InsP_3$/BM (+)-**3** *did* respond to ATP, showing that $InsP_3$Rs were present (Fig. 3a) and that the cells were viable. Further studies indicate that the products formed from BM-ester hydrolysis have no Ca^{2+}-mobilizing ability. These results are as would be predicted from the established pharmacology of D- and L-$InsP_3$[37] and therefore our data indicate that D-$InsP_3$/BM (−)-**3** exerts its Ca^{2+}-releasing actions via activation of $InsP_3$Rs. This material has proved also useful in studies conducted in a range of biological systems.[12,13,21–27]

Conclusions

In conclusion we have synthesized both the D- and the L-enantiomers of $InsP_3$/BM, using two different approaches for D-$InsP_3$/BM (−)-**3**. It has been shown that the intracellular Ca^{2+} releasing ability of $InsP_3$/BM resides in the D-enantiomer, while the L-enantiomer displays little biological activity. This supports the theory that the membrane permeant forms of $InsP_3$ exert their Ca^{2+}-mobilizing effects through activation of $InsP_3$Rs, rather than some non-specific action on the cell. The D- and L-enantiomers of $InsP_3$/BM are likely to remain important tools for those studying the role of $InsP_3$Rs in Ca^{2+} signalling.

Experimental

^{1}H NMR spectra were recorded on Bruker DPX-250 (250 MHz), DRX-400 (400 MHz) and DRX-500 (500 MHz) instruments, using deuterated chloroform as reference and internal deuterium lock. The chemical shift data for each signal are given in units of δ in parts per million (ppm) relative to tetramethylsilane (TMS) where $\delta_{TMS} = 0$. ^{13}C NMR spectra were recorded on Brucker DPX-250 (62.5 MHz), DRX-400 (100 MHz), and DRX-500 (125 MHz) instruments using an internal deuterium lock and proton decoupling. The chemical shift data for each signal are given as δ in units of parts per million (ppm) relative to tetramethylsilane where $\delta_{TMS} = 0$. In some cases the multiplicity of each signal was determined by an attached proton test experiment. ^{31}P NMR spectra were recorded on a Bruker DRX-400 (162 MHz) or Bruker DRX-500 (202 MHz) instrument with proton decoupling. The chemical shift data for each signal are given as δ in units of ppm relative to external 85% H_3PO_4.

Infrared (IR) spectra were recorded on a Perkin–Elmer 1310 spectrometer. The sample was prepared as a solution in the indicated solvent. Calibration was made relative to polystyrene at 1603 cm^{-1}.

Mass spectra were recorded at the EPSRC Mass Spectroscopy Centre, University of Wales, Swansea, or at the Department of Chemistry, University of Cambridge. Microanalyses were carried out by the staff of the University Chemical Laboratory, Cambridge, Analytical Department.

Melting points were determined using Reichert Hotstage melting point apparatus and are uncorrected. Kugelrohr bulb-to-bulb distillations were carried out using a Büchi GKR-51 machine. Boiling points are the actual oven temperatures.

Optical specific rotations were measured using a Perkin Elmer 241 polarimeter, in a cell of 1 dm path length. The concentration (c) is expressed in g/100 cm^3 (equivalent to g/0.1 dm^3) and values are stated in implied units of 10^{-1} deg cm^2 g^{-1}.

Analytical thin layer chromatography (TLC) was carried out on precoated 0.25 mm thick Merck 60 F_{254} silica gel plates. Visualization was by absorption of UV light, thermal development after treatment with basic potassium permanganate solution or an ethanolic solution of phosphomolybdic acid. Flash chromatography was carried out using Merck Kieselgel 60 (230–400 mesh) under a pressure of compressed air (typically 0.2–0.3 bar).

Dry THF was distilled from potassium in a recycling still using benzophenone ketyl as an indicator. Reagents were purified and dried where necessary by standard techniques. Ether refers to diethyl ether and *n*-hexane is referred to as hexane. Where appropriate and if not stated non-aqueous reactions were carried out under an atmosphere of argon. In vacuo refers to the removal of volatile solvents using a rotary evaporator. Room temperature (RT) refers to ambient laboratory temperature.

(−)-1D-1,4,5-Tris-O-*allyl-2,3,6-tris*-O-*benzyl*-myo-*inositol (−)-**5***

Into a suspension of sodium hydride (21.0 mg, 60% dispersion in mineral oil, 0.524 mmol) in DMF (2 mL) at 0°C was cannulated a solution of the alcohol (−)-**4** (210 mg, 0.437 mmol) in DMF (4 mL). The suspension was stirred at 0°C for 15 min before benzyl bromide (89.6 mg, 62.4 μL, 0.524 mmol) was added dropwise and the suspension stirred for 16 h at RT. The reaction was quenched by the addition of methanol (0.2 mL). The volatile components were removed in vacuo. The residue was partitioned between diethyl ether (10 mL) and water (5 mL) and the organic layer was separated. The aqueous layer was extracted with diethyl ether (10 mL) and the combined organic extracts washed with brine (5 mL), dried ($MgSO_4$), filtered, and the solvent removed in vacuo. Silica gel column chromatography of the residue, eluting with hexane and Et_2O (6:1 then 2:1), gave the title compound (−)-**5** (202 mg, 81%) as a colourless oil. R_f 0.57 (Et_2O/hexane 3:1). $[\alpha]_D^{20} = -3.5$ (*c* 1.12, $CHCl_3$) [lit.,[38] $[\alpha]_D^{30} = -2.5$ (*c* 1.6, $CHCl_3$)]. δ_H (250 MHz; $CDCl_3$) 7.42–7.23 (15H, m, Ph), 6.04–5.85 (3H, m, $CH_2CH{=}CH_2$), 5.32–5.12 (6H, m, $CH_2CH{=}CH_2$), 4.86 (2H, s, CH_2-Ph), 4.82 (2H, ABq, ^2J 10.3, CH_2-Ph), 4.63 (2H, ABq, ^2J 11.9, CH_2-Ph), 4.36–4.31 (4H, m, $CH_2CH{=}CH_2$), 4.09–4.07 (2H, m, $CH_2CH{=}CH_2$), 3.99–3.97 (1H, m, inositol ring H), 3.92 (1H, apparent t, *J* 9.6, inositol ring H), 3.87 (1H, t, *J* 9.6, inositol ring H), 3.28–3.17 (3H, m, 3 × inositol ring H). These data are in agreement with the literature values.[38]

(+)-1L-1,4,5-Tris-O-*allyl-2,3,6-tris*-O-*benzyl*-myo-*inositol (+)-**5***

(+)-1L-1,4,5-Tris-*O*-allyl-2,3,6-tris-*O*-benzyl-*myo*-inositol (+)-**5** was prepared in a manner similar to that described for its antipode, from (+)-**4**. Yield 295 mg (73%). $[\alpha]_D^{20} = +3.0$ (*c* 1.05, $CHCl_3$).[39] All other spectroscopic and analytical data were identical to that reported for the enantiomeric material, (−)-**5**.

(+)-1D-2,3,6-Tris-O-*benzyl*-myo-*inositol (+)-**6** (Method 1)*

To a solution of the allyl ether (−)-**5** (198 mg, 0.347 mmol) and Hünig's base (40.3 mg, 54 μL, 0.312 mmol) in ethanol–toluene–water (7:3:1, 11 mL) was added Wilkinson's catalyst [$(Ph_3P)_3RhCl$] (144 mg, 0.156 mmol) and the solution heated under reflux for 2 h, then cooled to RT. The mixture was diluted with ethyl acetate (5 mL) and insoluble material was removed by filtration through Hyflo, under vacuum. To the filtrate was added water (10 mL) and ethyl acetate (15 mL). The layers were separated and the aqueous phase was extracted with ethyl acetate (10 mL). The combined organic layers were washed with brine (10 mL), dried ($MgSO_4$), filtered, and evaporated in vacuo. The residue was dissolved in MeOH (7 mL) and dichloromethane (14 mL) and acetyl chloride (20.4 mg, 18.5 μL, 0.260 mmol) was added. The solution was stirred at RT for 70 min. Et_3N (79 mg, 109 μL, 0.781 mmol) was added and the mixture was concentrated in vacuo. Silica gel column chromatography eluting with hexane and ethyl acetate (2:1 then 1:1) afforded the triol (+)-**6** (117 mg, 75%) as an off-white solid. R_f 0.19 (Et_2O/hexanes 1:1). mp 119–120°C (from ethyl acetate) [lit.,[28] mp 117–119°C; lit.,[29] mp 117–119°C; lit.,[30] mp 122–123°C; lit.,[38] 117–119°C]. $[\alpha]_D^{20} = +12.3$ (*c* 0.40, $CHCl_3$) [lit.,[28] $[\alpha]_D^{16} = +15.5$ ($CHCl_3$); lit.,[29] $[\alpha]_D = +12.4$ (*c* 0.80, $CHCl_3$); lit.,[30] $[\alpha]_D = +10.3$ (*c* 1.73, $CHCl_3$); lit.,[38] $[\alpha]_D^{16} = +15.5$ (*c* 1.0, $CHCl_3$)]. δ_H (250 MHz; $CDCl_3$) 7.37–7.33 (15H, m, Ph), 4.83 (2H, ABq, ^2J 11.6), 4.87 (2H, ABq, ^2J 11.8), 4.64 (2H, ABq, ^2J 11.7), 4.09 (1H, apparent t, *J* 2.5, inositol ring H), 4.02 (1H, dt, *J* 9.6, 1.9, inositol ring H), 3.69 (1H, apparent t, *J* 9.2 inositol ring H), 3.44–3.58 (2H, m, 2 × inositol ring H), 3.30 (1H, dd, 1H, *J* 9.7, 2.4, inositol ring H), 2.61–2.58 (2H, m, O*H*), 2.33 (1H, d, *J* 6.5, O*H*). These data are in agreement with the literature values.[28–30,38]

(+)-1D-2,3,6-Tris-O-*benzyl*-myo-*inositol (+)-**6** (Method 2)*

(−)-5-*O*-Allyl-2,3,6-tris-*O*-benzyl-1-*O*-(4-methoxybenzyl)-*myo*-inositol (−)-**9** (300 mg, 0.47 mmol),[19] Wilkinson's catalyst (131.3 mg, 0.14 mmol) and Hünig's base (61.2 mg, 82.4 μL, 0.47 mmol) were dissolved in ethanol (15 mL) and heated under reflux. As the starting material and product have the same TLC R_f value the reaction progress was monitored by ^1H NMR spectroscopy. The resonance for the alkene CH proton is observed at δ_H ~5.9 ppm in the starting material and δ_H ~6.3 ppm in the enol ether. The reaction was seen to be complete by ^1H NMR analysis after 1.5 h. The reaction mixture was filtered through Celite and then concentrated under reduced pressure. The resulting oil was suspended in water (30 mL) and extracted with ethyl acetate (4 × 20 mL). The combined organic fractions were washed with saturated brine (20 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. This afforded crude 1-*O*-(4-methoxybenzyl)-5-*O*-propenyl-2,3,6-tris-*O*-benzyl-*myo*-inositol as an oil which was used, without purification. (−)-1-*O*-(4-Methoxybenzyl)-5-*O*-propenyl-2,3,6-tris-*O*-benzyl-*myo*-inositol (assumed 300 mg) was dissolved in methanol (20 mL) and concentrated HCl (1 mL) was added. The solution was heated under reflux in air for 2 h, by which time the reaction was adjudged to be complete by TLC analysis. The solution was cooled and solid $NaHCO_3$ (840 mg, 10 mmol, 1 equiv wrt HCl) was added to neutralize the HCl. The resulting mixture was filtered and the methanol removed from the filtrate under reduced pressure. Purification using silica gel column chromatography eluted with ethyl acetate and hexane (2:3) furnished (−)-2,3,6-tris-*O*-benzyl-*myo*-inositol (+)-**6** (147.8 mg, 66% yield) as an off-white solid. R_f 0.62 (ethyl acetate). mp 118–119°C (from ethyl acetate) [lit.,[28] mp 117–119°C; lit.,[29] mp 117–119°C; lit.,[30] mp 122–123°C]. $[\alpha]_D^{25} = +12.1$ (*c* 0.88, $CHCl_3$) [lit.,[28] $[\alpha]_D^{16} = +15.5$ ($CHCl_3$); lit.,[29] $[\alpha]_D = +12.4$ (*c* 0.80, $CHCl_3$); lit.,[30] $[\alpha]_D = +10.3$ (*c* 1.73, $CHCl_3$)]. δ_H (500 MHz; $CDCl_3$) 7.41–7.30 (15H, m, Ph), 4.96 (1H, d, J_{AB} 11.4, CH_AH_BPh), 4.90 (1H, d, $J_{A'B'}$ 11.4, $CH_{A'}H_{B'}Ph$), 4.86 (1H, d, $J_{A'B'}$ 11.4, $CH_{A'}H_{B'}Ph$), 4.74 (1H, d, $J_{A''B''}$ 11.7, $CH_{A''}H_{B''}Ph$), 4.71 (1H, d, J_{AB} 11.4, CH_AH_BPh), 4.61 (1H, d, $J_{A''B''}$ 11.7, $CH_{A''}H_{B''}Ph$), 4.10 (1H, apparent t, *J* 2.6, inositol ring H), 4.03 (1H, td, *J* 9.4, 1.9, inositol ring H), 3.70 (1H, apparent t, *J* 9.3, inositol ring H), 3.58–3.54 (1H, m, inositol ring H), 3.50 (1H, td, *J* 9.1, 2.1, inositol ring H), 3.32 (1H, dd, *J* 9.7, 2.4, inositol ring H), 2.60 (2H, apparent dd, *J* 8.5, 2.1, 2 × O*H*), 2.35 (1H, d, *J* 6.6, O*H*). These data are in agreement with the literature values.[28–30]

(+)-1D-2,3,6-Tris-O-*benzyl*-myo-*inositol 1,4,5-Trisphosphate Hexakis-(2-cyanoethyl) Ester (+)-**7***

N,*N*-Diisopropylamino bis-(2-cyanoethoxy)phosphine (633 mg, 2.33 mmol) and 1*H*-tetrazole (196.2 mg, 2.8 mmol) were dissolved in dry dichloromethane (4 mL) and stirred for 1 h at RT. (+)-2,3,6-Tris-*O*-benzyl-*myo*-inositol (+)-**6** (140 mg, 0.31 mmol) was dissolved in dry dichloromethane (3 mL) and cannulated into the stirred phosphite solution and washed in with dry dichloromethane (3 mL). The resulting solution was stirred for 45 h when a new product and no starting material were observed by TLC analysis. The solution was cooled to −78°C and solid *m*CPBA (483 mg, 2.8 mmol) was added. The resulting mixture was stirred for 1 h at −78°C then overnight at RT. Formation of a more polar spot than previously observed was seen by TLC analysis. The organic layer was washed with a saturated aqueous solution of $NaHSO_3$ (10 mL), a saturated aqueous solution of $NaHCO_3$ (20 mL) then water (20 mL). The combined aqueous layers were washed with dichloromethane (3 × 30 mL). The combined organic layers were washed with saturated brine (20 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification using silica gel column chromatography eluted with ethyl acetate then ethyl acetate and methanol (90:10) afforded pure *(+)-1D-2,3,6-tris*-O-*benzyl*-myo-*inositol 1,4,5-trisphosphate hexakis-(2-cyanoethyl) ester (+)-**7*** (291 mg, 93% yield) as a clear colourless resin. R_f 0.1 (ethyl acetate). $[\alpha]_D^{[26]} = +3.7$ (*c* 0.6, $CHCl_3$). ν_{max} ($CHCl_3$)/cm^{-1} 2969 (w) (CH), 2937 (w) (CH), 2255 (w) (CN), 1717 (w), 1498 (w) (Ph), 1471 (w) (Ph), 1456 (w) (Ph), 1279 (s) (*P*=O), 1028 (s), 752 (m) (5 adjacent Ph), 700 (m) (5 adjacent Ph). δ_H (500 MHz; $CDCl_3$) 7.46–7.42 (15H, m, Ph), 4.97–4.75 (6H, m, Ph-CH_2),

4.58–3.96 (17H, m, 6 × PO-CH_2 and 5 × inositol ring H), 3.63 (1H, dd, *J* 9.9, 2.0, 3-H), 2.70–2.21 (12H, m, CH_2-CN). δ_C (125 MHz; $CDCl_3$) 137.9, 137.8, 137.1, 128.6, 128.5, 128.4, 128.2, 128.0, 127.8, 127.8, 127.7, 126.7, 117.0 (CN), 116.8 (CN), 116.6 (CN), 116.5 (CN), 116.4 (CN), 116.3 (CN), 79.3, 78.2–78.1 (2C, m, 2 × inositol ring C), 77.6–77.5 (2C, m, 2 × inositol ring C), 75.6, 74.8 (inositol ring C), 74.8, 72.5, 63.0 (1C, d, J_{CP} 5.5, PO-CH_2), 62.8 (1C, d, J_{CP} 5.5, PO-CH_2), 62.5–62.4 (2C, m, PO-CH_2), 62.3–62.2 (2C, m, PO-CH_2), 19.6 (4C, m, PO-CH_2CH_2), 19.1 (1C, d, J_{CP} 7.8, PO-CH_2CH_2), 19.0 (1C, d, J_{CP} 8.1, PO-CH_2CH_2). δ_P (202 MHz; $CDCl_3$) −2.7, −2.5 (1:2). *m/z* (ES+, NH_3) 1031.4 [100, $(M + Na)^+$], 1026.5 [35, $(M + NH_4)^+$], 1009.4 [50, $(M + H)^+$], 956.4 (10), 829.3 (20), 739.2 (20), 310.2 (15), 235 (15), 181.1 (60), 143.7 (60), 90.8 (70). The appropriate data are in agreement with literature values for the racemic compound.[4]

*(−)-1L-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-Trisphosphate Hexakis-(2-cyanoethyl) Ester (−)-**7***

(−)-1L-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis-(2-cyanoethyl) ester (−)-**7** was prepared in a manner similar to that described for its antipode, from (−)-**6**. Yield 246 mg (92%). $[\alpha]_D^{20} = -4.7$ (*c* 1.6, $CHCl_3$). All other spectroscopic and analytical data were identical to that reported for the enantiomeric material, (+)-**7**.

*(−)-1D-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate Hexakis(butyryloxymethyl) Ester (−)-**8***

(+)-1D-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis-(2-cyanoethyl) ester (+)-**7** (47.5 mg, 0.05 mmol) was dissolved in dry dichloromethane (1 mL) under argon and triethylamine (47.6 mg, 65.4 μL, 0.47 mmol, 10 equiv) was added. After stirring for 16 h at RT the solvent was removed in vacuo. Freshly distilled bromomethyl butyrate (255 mg, 1.41 mmol) was added to the residue and mixture was placed under an atmosphere of argon. Dry acetonitrile (2 mL) followed by Hünig's base (182.6 mg, 246.0 μL, 1.41 mmol) were added and the resulting solution was stirred for 48 h. A reaction was adjudged to have occurred by TLC analysis. The solvent was removed in vacuo and purification by silica gel column chromatography eluted with ethyl acetate and hexane (40:60 then 50:50) furnished *(−)-1D-2,3,6-tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis(butyryloxymethyl) ester (−)-**8*** (29.4 mg, 48% yield) as a clear colourless oil. R_f 0.14 (ethyl acetate/hexane, 50:50). $[\alpha]_D^{20} = -3.9$ (*c* 1.6, $CHCl_3$). ν_{max} (neat film)/cm^{-1} 2964 (m) (CH), 2932 (m) (CH), 2877 (m) (O-CH_2-O), 1763 (s) (CO), 1458 (m) (Ph), 1416 (m) (Ph), 1364 (m), 1283 (m) (P-O-aryl), 1262 (m) (*P*=O), 1151 (s) (C–O stretch ether), 1099 (m) (P-O-alkyl), 963 (s). δ_H (500 MHz; $CDCl_3$) 7.41–7.23 (15H, m, Ph), 5.65–5.58 (4H, m, O-CH_2-O), 5.51–5.38 (7H, m, O-CH_2-O), 5.31–5.27 (1H, m, O-CH_2-O), 4.89–4.79 (3H, m, CH_2-Ph and 1 × inositol ring H), 4.74–4.71 (2H, m, CH_2-Ph), 4.66 (1H, d, J_{AB} 11.5, CH_AH$_B$-Ph), 4.62 (1H, d, J_{AB} 11.5, CH_AH$_B$-Ph), 4.41 (1H, apparent q, *J* 9.3, inositol ring H), 4.33 (1H, t, *J* 2.3, inositol ring H), 4.30–4.26 (1H, m, inositol ring H), 4.03 (1H, t, *J* 9.6, inositol ring H), 3.47 (1H, dd, *J* 9.9, 2.2, inositol ring H), 2.31–2.22 (12H, m, O=C-CH_2), 1.65–1.55 (12H, m, O=C-CH_2-CH_2), 0.94–0.82 (18H, m, CH_3). δ_C (125 MHz; $CDCl_3$) 171.8, 171.8, 171.7, 137.9, 137.7, 137.0, 128.8, 128.5, 128.3, 128.2, 127.9, 127.8, 127.7, 127.7, 127.5, 82.9 (2C, m, O-CH_2-O), 82.6–82.5 (3C, m, O-CH_2-O), 82.4 (1C, d, J_{CP} 4.5, O-CH_2-O), 79.2, 78.3 (1C, d, J_{CP} 5.5, inositol ring C), 78.2–78.1 (1C, m, inositol ring C), 77.4–77.3 (2C, m, 2 × inositol ring C), 75.3, 75.1, 74.9 (inositol ring C), 72.6, 35.6–35.5 (6C, m, O=C-CH_2), 17.9, 13.5–13.4 (6C, m, O=C-CH_2-CH_2-CH_2). δ_P (202 MHz; $CDCl_3$) −4.7, −4.3, −4.0. *m/z* (ES+, NH_3) 1313.6 [60, $(M + Na)^+$], 1308.6 [100, $(M + NH_4)^+$], 932.5 (100), 860.3 (70), 739.5 (85), 663.4 (70), 491.2 (100), 42.1 (100).

*(+)-1L-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-Trisphosphate Hexakis(butyryloxymethyl) Ester (+)-**8***

*(+)-1L-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis (butyryloxymethyl) ester (+)-**8*** was prepared in a manner similar to that described for its antipode, from (−)-**7**. Yield 76 mg (55%). $[\alpha]_D^{20} = +3.5$ (*c* 1.9, $CHCl_3$). All other spectroscopic and analytical data were identical to that reported for the enantiomeric material, (−)-**8**.

*(−)-1D-*myo*-Inositol 1,4,5-Trisphosphate Hexakis(butyryloxymethyl) Ester (−)-**3** (D-InsP$_3$/BM)*

(−)-1D-2,3,6-Tris-*O*-benzyl-*myo*-inositol 1,4,5-trisphosphate hexakis (butyryloxymethyl) ester (25.8 mg, 20.0 μmol) (−)-**8** was dissolved in glacial acetic acid (2 mL) under argon. Palladium bis(trifluoromethylacetate) (19.3 mg, 58.1 μmol) and palladium diacetate (38.6 mg, 171.9 μmol) were added and the mixture was stirred at 10°C for 2.5 h under an atmosphere of hydrogen. The suspension was filtered through Hyflo, which was then washed with glacial acetic. The resulting solution was diluted with Milli-Q purified water (1 mL) and lyophilized to afford *(−)-1D-*myo*-inositol 1,4,5-trisphosphate hexakis (butyryloxymethyl) ester (−)-**3*** (17.8 mg, 91% yield) as a colourless solid. $[\alpha]_D^{25} = -10.8$ (*c* 0.46, MeOH). ν_{max} (neat film)/cm^{-1} 3727 (w) (OH), 2925 (s) (CH), 2855 (m) (O-CH_2-O), 1733 (s) (CO), 1465 (m), 1380 (m), 1262 (m) (*P*=O), 997 (s). δ_H (400 MHz; MeOD) 5.71–5.64 (12H, m, O-CH_2-O), 4.62 (1H, q, *J* 9.4, inositol ring H), 4.38–4.24 (2H, m, 2H, inositol ring H), 4.18 (1H, apparent t, *J* 2.5, inositol ring H), 4.02 (1H, apparent t, *J* 9.5, inositol ring H), 3.68 (1H, dd, *J* 9.7, 2.5, inositol ring H), 2.42–2.37 (12H, m, C(O)CH_2), 1.65 (12H, hx, *J* 7.4, C(O)CH_2-CH_2), 0.96 (18H, t, *J* 7.4, CH_3). δ_P (162 MHz; MeOD) −4.1, −3.8, −3.6. *m/z* (ES+) 1043.8 [20, $(M + Na)^+$], 960.4 (60), 471.7 (80), 417.0 (65), 116.6 (50), 81.3 (70), 74.1 (100). The appropriate data are in agreement with literature values for the racemic compound.[4]

*(+)-1L-*myo*-Inositol 1,4,5-Trisphosphate hexakis(butyryloxymethyl) Ester (+)-**3** (L-InsP$_3$/BM)*

*(+)-1L-*myo*-Inositol 1,4,5-trisphosphate hexakis(butyryloxy-methyl) ester (+)-**3*** was prepared in a manner similar to that described for its antipode, from (+)-**8**. Yield 26 mg (88%). $[\alpha]_D^{[20]} = +8.3$ (*c* 1.4, $CHCl_3$). All other spectroscopic and analytical data were identical to that reported for the enantiomeric material, (−)-**3**.

*Methylene Dibutyrate **11***

Butyric acid (5.3 g, 5.5 mL, 60.2 mmol) was added to a 2 M solution of sodium hydroxide (4.8 g, in 60 mL of water) and stirred for 30 min under air. Tetrabutylammoniumhydrogen sulfate (20.4 g, 60.1 mmol) was added, heat was evolved and the resulting solution was stirred for 30 min. The aqueous solution was extracted with dichloromethane (4 × 100 mL). The combined organic fractions were dried ($MgSO_4$), filtered, and heated under reflux for 2 days. The dichloromethane was removed by distillation and the resulting oil was transferred to a smaller vessel. This oil was purified by vacuum distillation, the fraction boiling at 43°C under 0.3 mmHg of pressure was pure methylene dibutyrate **11** (3.15 g, 55.7% yield) as a clear colourless oil. δ_H (250 MHz; $CDCl_3$) 5.75 (2H, s, OCH_2O), 2.34 (4H, t, *J* 7.4, CH_2C=O), 1.67 (4H, sx, *J* 7.4, CH_3CH_2), 0.95 (6H, t, *J* 7.4, CH_3CH$_2$). These data are in agreement with the literature values.[31]

*Bromomethyl Butyrate **12***

Methylene dibutyrate **11** (1.02 g, 5.4 mmol, 1 equiv), trimethylsilyl bromide (1.08 g, 0.93 mL, 7.1 mmol, 1.3 equiv) and zinc(II) bromide (61 mg, 0.27 mmol, 0.05 equiv) were stirred together overnight. The reaction was adjudged to be incomplete by ^{1}H NMR analysis, therefore trimethylsilyl bromide (0.54 g, 0.47 mL, 3.6 mmol, 0.7 equiv) was added and the resulting solution was stirred for a further 24 h when the reaction was adjudged to be complete by ^{1}H NMR analysis. The solution was diluted with ether (20 mL) and a 1 M aqueous HCl solution was added (10 mL) and the mixture was stirred for 10 min. The aqueous phase was removed and the organic fraction was stirred with a saturated solution of Na_2CO_3 (20 mL) for 30 min. The organic fraction was washed with water (10 mL) and a saturated brine solution (10 mL), dried ($MgSO_4$), filtered, and the ether was removed under reduced pressure at RT. The resulting oil was adjudged to consist mainly of the required product contaminated with ∼10% of methylene butyrate. The oil was further purified by Kugelrohr distillation, the fraction which boiled at

90°C under 5 mmHg of pressure was pure bromomethyl butyrate **12** (472.2 mg, 48.2% yield) as a clear colourless oil. δ_H (250 MHz; $CDCl_3$) 5.81 (2H, s, CH_2Br), 2.36 (2H, t, *J* 7.4, $CH_2C{=}O$), 1.69 (2H, sx, *J* 7.4, CH_3CH_2), 0.97 (3H, t, *J* 7.4, CH_3CH_2).[32]

Cell Culture

Human embryonic kidney cells (HEK-293) were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2 mM L-glutamine in a humidified 95% air, 5% CO_2 incubator. All experimental procedures were carried out at room temperature (20–22°C). Prior to imaging, the culture medium was replaced with an extracellular medium (EM) containing (mM): NaCl, 121; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; $NaHCO_3$, 6; D-glucose, 5.5; Hepes, 25; pH 7.3.

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