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A MEMBRANE-PERMEANT, BIOACTIVATABLE DERIVATIVE OF INS(1,3,4)P_3 AND ITS EFFECT ON Cl^--SECRETION FROM T_{84} CELLS

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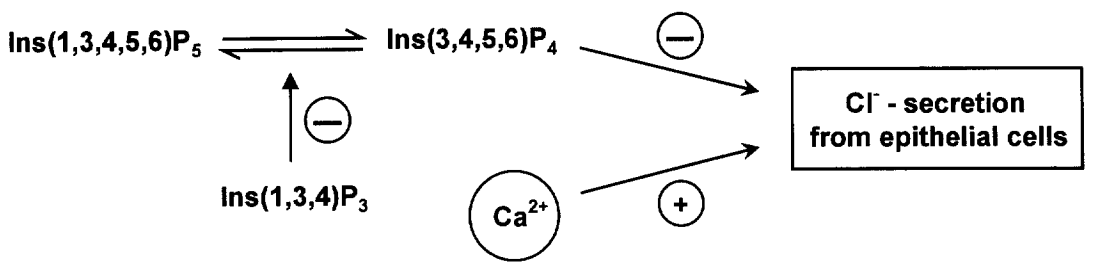
Abstract: The synthesis of *rac*-2,5,6-tri-*O*-butyryl-*myo*-inositol 1,3,4-trisphosphate hexakis(acetoxymethyl) ester [Bt_3-Ins(1,3,4)P_3/AM, **1**], a membrane-permeant derivative of *myo*-inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3] is reported. **1** inhibited calcium-mediated chloride secretion of T_{84} cells, suggesting a regulatory link of Ins(1,3,4)P_3 and the biosynthesis of the known inhibitor *myo*-inositol 3,4,5,6-tetrakisphosphate.

Chloride secretion of epithelial cells participates in a wide range of physiological and pathological activities,[1] the latter including diseases like cystic fibrosis [2] and secretory diarrhea.[3] In particular the regulation of calcium mediated chloride secretion (CaMCS) has been recently investigated resulting in the finding that receptor-regulated signaling not only increased calcium levels mediated by *myo*-inositol 1,4,5-trisphosphate but also gave rise to intracellular levels of *myo*-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P_4].[4] Ins(3,4,5,6)P_4 was subsequently identified as an inhibitor of CaMCS by elevating intracellular Ins(3,4,5,6)P_4 levels with the help of a membrane-permeant, bioactivatable derivative of Ins(3,4,5,6)P_4 (mimicking receptor occupation).[5] This effect of Ins(3,4,5,6)P_4 was recently shown to directly block calcium-activated chloride channels.[6,7] It is currently unknown how receptor occupation elevates Ins(3,4,5,6)P_4 levels, but Menniti *et al.* suggested that the biosynthesis of Ins(3,4,5,6)P_4 relies on an equilibrium between *myo*-inositol 1,3,4,5,6-pentakisphosphate and Ins(3,4,5,6)P_4 levels which are regulated by a 1-phosphatase and a 1-kinase, respectively (Scheme 1).[8] More recently, it was found that the partially-purified 1-kinase was potently inhibited by *myo*-inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3],[9] opening the possiblity of a regulatory link between receptor occupation, phosphatidyl 4,5-bisphosphate breakdown, and elevated levels of Ins(3,4,5,6)P_4.



Scheme 1: A possible physiological function of Ins(1,3,4)P_3: inhibition of the 1-kinase leads to elevated levels of Ins(3,4,5,6)P_4 and subsequently to an inhibitory effect on calcium mediated chloride secretion.

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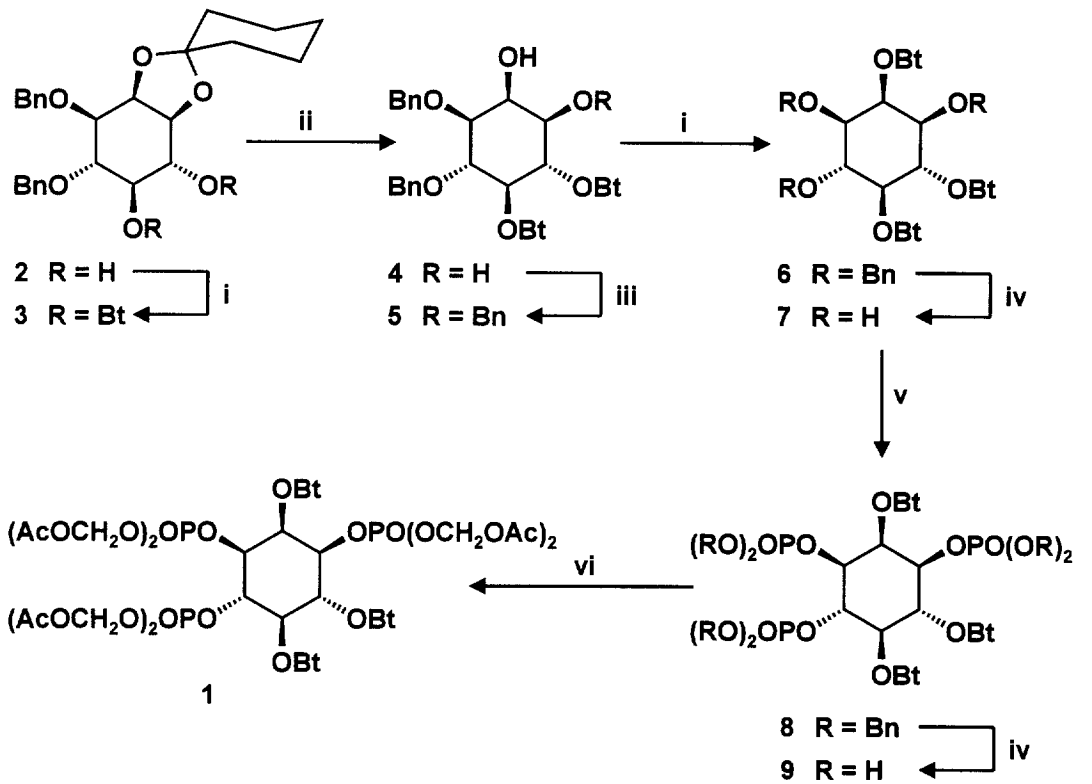
To facilitate investigation of this hypothetical physiological role of Ins(1,3,4)P_3 we here report the synthesis of a membrane-permeant Ins(1,3,4)P_3 derivative with bioactivatable butyrates and acetoxymethyl esters (AM-esters) masking the hydroxy groups and negatively charged phosphates, respectively.

Results and Discussion

The synthetic pathway differed from those for the total synthesis[10-12] of Ins(1,3,4)P_3 in a way that allowed the regioselective introduction of butyrates as being stable protecting groups during synthesis and bioactivatable groups in the final product.



Scheme 2. **i** Bt_2O, DMAP, pyr., **ii** TFA, MeCN, H_2O, **iii** *a* Bu_2SnO, toluene, refl., *b* BnBr, CsF, DMF, **iv** Pd / C (10%), AcOH, **v** *a* $(BnO)_2PNiPr_2$, tetrazole, MeCN, *b* AcOOOH, - 40°C, **vi** AMBr, DIEA, MeCN.

The reaction sequence to **1** (Scheme 2) started with 3,4-di-*O*-benzyl-1,2-*O*-cyclohexyliden-*myo*-inositol (**2**), which was prepared by following a slightly modified version of the procedure of Angyal *et al.*.[13] The two hydroxyl groups were esterified with butyric anhydride in pyridine containing traces of 4-dimethylamino pyridine (DMAP) to give the fully protected compound **3**. Removal of the ketale by trifluoroacetic acid (40%) in MeCN with traces of water (1%) yielded roughly quantitative amounts of the diol **4**. The alkylation to the benzyl group at the 1-position in the presence of the esters was achieved via a cyclic dibutyl tin intermediate which was readily opened by benzyl bromide without the need for base or acid.[14] The resulting compound **5** was

butyrylated to give the fully protected compound **6** which was catalytically hydrogenated to give the tributyrate **7**. The total yield of the regioselectively protected presursor **7** from **2** was 62%. Phosphates were introduced by a classical phosphite approach[15] with subsequent oxidation to give the fully protected 1,3,4-tris(dibenzyl)phosphate **8**. Benzyl protecting groups were removed by hydrogenolysis and the resulting free acid **9** was treated with acetoxymethyl bromide in acetonitrile in the presence of di*iso*propylethyl amine as a sterically hindered base. The hexakis(acetoxymethyl) ester **1** was isolated by extraction of the crude product with toluene and subsequent purification on a preparative reversed-phase column (50 x 250 mm, 10 µm, RP-18, Merck, 73% MeOH). The overall isolated yield from **7** was 42%.

We have shown before that preincubation of cells of the human epithelial cell line T_{84} with 1,2-di-*O*-butyryl-Ins(3,4,5,6)P_4/AM resulted in a significant increase in intracellular Ins(3,4,5,6)P_4 levels which coincided with inhibition of transepithelial Cl^--transport.[5]

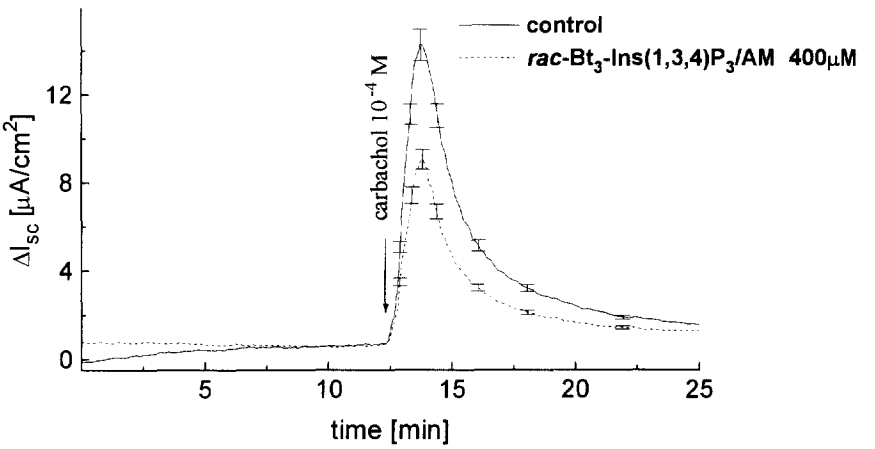


Figure 1: Cl^--secretion of confluent monolayers of T_{84} cells measured as short circuit current (ΔI_{SC}). Cells were incubated with **1** (400 µM) dissolved in DMSO / Pluronic (5%) for 30 min prior to mounting the cells in Ussing chambers. Control cells were treated in a way that the final DMSO concentration did not exceed 0.05%. Data reflect 4 second monitoring. The difference in the mean peak ΔI_{sc} is significant.
Control: 15.2 ± 2.8; Bt_3-Ins(1,3,4)P_3/AM (**1**): 7.9 ±1.6. $p < 0.04$, unpaired, two-sided, students t-test. n = 8.

Here, we performed similar experiments by treating confluent monolayers of T_{84} cells with the membrane-permeant Ins(1,3,4)P_3 derivative **1** (400µM) for 30 min. After mounting the cells in modified Ussing chambers, Cl^--secretion measured as short circuit current (ΔI_{sc}) across the monolayer was monitored as described before.[5] After 12.5 min carbachol was added to induce CaMCS (Figure 1). The size of the inhibition was roughly equivalent to that observed following incubations with comparable amounts of 1,2-di-*O*-butyryl-Ins(3,4,5,6)P_4/AM.[16]

The results shown hint towards a physiological role of Ins(1,3,4)P_3 in epithelial cells. A direct inhibitory effect on Cl^--secretion of T_{84} cells is unlikely.[6] This supports the hypothesis that Ins(1,3,4)P_3 regulates the equilibrium of $InsP_5$ 1-phosphatase and Ins(3,4,5,6)P_4 1-kinase as was proposed in Scheme 1.[9] However, only inositol phosphate mass analysis of T_{84} cell lysates will prove whether the proposed effect on intracellular Ins(3,4,5,6)P_4 levels is responsible for the inhibitory effect on Cl^--secretion. Furthermore, synthesis and application of

enantiomerically pure membrane-permeant derivatives of Ins(1,3,4)P_3 should rule out the possibility that the enantiomeric Ins(1,3,6)P_3 derivative is responsible for the physiological effect. In the future, membrane-permeant bioactivatable derivatives of the different inositol phosphates occuring in living cells might become prime tools to investigate the unknown function of many of these potential signaling molecules.

Materials and Methods

Measurement of short-circuit current in Ussing chambers, which totally reflect secretion of chloride ions, were performed as described before.[5]

All products gave satisfactory NMR and mass spectroscopy data including high-resolution FAB-MS-data.

Selected data from **1**: ^{1}H NMR ([D]$_8$ toluene, 360 MHz): δ 0.83 (3 H, t, J = 7.28 Hz, CH_3), 0.92 (3 H, t, J = 7.28 Hz, CH_3), 0.97 (3 H, t, J = 7.48 Hz, CH_3), 1.50-1.60 (2H, m, β-CH_2), 1.67-1.76 (4 H, m, 2 × β-CH_2), 1.79-1.96 (18 H, 6 × s, 6 × OAc), 2.08-2.13 (2 H, m, α-CH_2), 2.33-2.47(2 H, m, α-CH_2), 2.52-2.61 (2 H, m, α-CH_2), 5.05 (1 H, ddd, J = 9.64, 9.64, 2.17 Hz, H-3), 5.09 (1 H, ddd, J = 9.64, 9.06, 9.06 Hz, H-4), 5.11 (1 H, ddd, J = 9.84, 9.84, 2.17 Hz, H-1), 5.50-5.83 (14 H, 6 × CH_2OAc, H-5, H-6), 6.21 (1 H, dd, J = 2.17, 2.17 Hz, H-2). ^{31}P NMR ([D]$_8$ toluene, ^{1}H-decoupled, 145.8 MHz): δ - 4.17 (1 P. s), - 4.05 (1 P. s), - 3.97 (1 P. s). MS: *m/z* (+ve ion FAB) 1063 [(M + H)$^+$, 10]. MS: *m/z* 991.199 (M - CH_2OAc + 2H)$^+$ (calcd. $C_{33}H_{54}O_{28}P_3$ 991.201).

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