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Synthesis and binding properties of cyclopentane analogues of *myo*-inositol 1,4,5-tris(phosphate)

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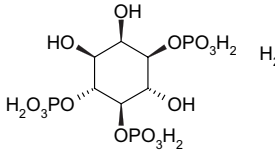
Abstract—Cyclopentanic analogues of *myo*-inositol 1,4,5-tris(phosphate) were synthesised starting from cyclopentadiene. The affinities of the trisphosphorylated derivatives for the Ins(1,4,5)P_3 receptors were equipotent to that of compound **4**, showing that the relative orientation of the functional groups, particularly of the hydroxyl, is not of prime importance in this series. The ^{31}P NMR titration curves show that the tris(phosphate) **5** behaves as the superimposition of an independent phosphate and a vicinal bis(phosphate).

1. Introduction

It is now well established that *myo*-inositol 1,4,5-tris(phosphate) (Ins(1,4,5)P_3) (**1**) acts as an intracellular second messenger. The binding of Ins(1,4,5)P_3 with its receptors (Ins(1,4,5)P_3R) induces the mobilisation of calcium from intracellular stores.[1–3]



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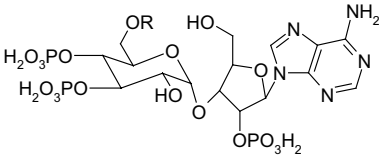
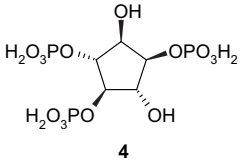


2 R = H
3 R = Ac



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The structure–activity relationship developed around this compound, especially towards the Ins(1,4,5)P_3R,[4–6] permitted Kozikowski to propose a pharmacophore model showing the crucial role of the vicinal bis(phosphate) in positions 4 and 5, the amplifying effect of the third phosphate in position 1, the major role played by the hydroxyl group in position 6 and the slight importance of the two hydroxyls in positions 2 and 3 (Fig. 1).[7,8]

The adenophostines **2** and **3** extracted from *Penicillium brevicompactum* are highly potent agonists of the Ins(1,4,5)P_3 receptors (10–30 times more potent than Ins(1,4,5)P_3).[9–12] These molecules contain two sugar backbones. Starting from the bisphosphorylated hexose moiety it is easy to imagine new Ins(1,4,5)P_3 analogues and such analogues were recently published.[13–16] Dreiding stereomodels show that the main functionalities of Ins(1,4,5)P_3 could be arranged around a cyclopentanic ring. Potter et al. have reported cyclopentanic analogues such as compound **4**. The relative configuration of the ring substituent strictly respect that of the parent Ins(1,4,5)P_3. This compound is a weak agonist for the Ins(1,4,5)P_3R (20% Ca^{++} mobilisation at 10^{-4} M) and possesses a weak affinity for this receptor (three orders of magnitude lower than the parent compound).[17]

Using ^{31}P NMR, it has been shown that the different functions of Ins(1,4,5)P_3 establish intramolecular

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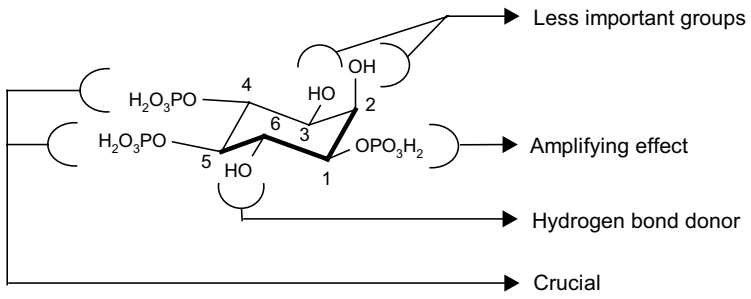
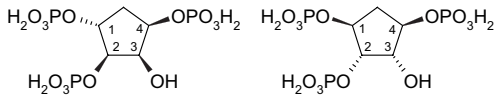


Figure 1. Ins(1,4,5)P_3 pharmacophore model proposed by Kozikowski.

cooperative effects especially between the phosphate groups. The cooperative effect seemed to be relayed by the neighbouring hydroxyls.[18–23] The same studies carried on compound **4** did not reveal the same type of cooperation.[24] The orientation of the functional groups induced by the five member ring could disrupt the interactions between the phosphate functions if the relative configuration of the Ins(1,4,5)P_3 backbone is too starkly transposed to the cyclopentanic ring. The interfunctional cooperation could be restored by changing the configuration of some of the backbone carbons.

We report here the synthesis and the properties of such cyclopentanic analogues of Ins(1,4,5)P_3. For these derivatives (**5**, **6**) we suppressed the hydroxyl in position 2, which did not seem important according to the Kozikowski's model.



5 **6**

For compound **5** the hydroxyl in position 3 corresponding to the hydroxyl in position 6 on Ins(1,4,5)P_3 was epimerised to see if this modification could restore the cooperative effects observed for Ins(1,4,5)P_3. For the analogue **6** we wanted to keep the *cis* relative orientation of the hydroxyl in position 3 and the phosphate in position 2. In a first attempt, the products were prepared as racemates. If their affinity justified it, the racemates could be resolved to identify the more active enantiomer.

2. Synthesis

Our synthetic scheme started with cyclopentadiene (**7**), which was treated with silver benzoate and iodine[25] giving three dibenzoate isomers; the *trans* 3-cyclopentene 1,2-dibenzoate (**8**), *trans* 2-cyclopentene 1,4-dibenzoate (**9**) and the *cis* 2-cyclopentene 1,4-dibenzoate (**10**) in a 15/20/20 proportion, respectively. The three isomers were separated by crystallisation and column chromatography. The *cis* dihydroxylation of the remaining double bond using potassium permanganate[26] led to the intermediates **11**–**13**. The isomer **12** proceeded by migration of the benzoate in position 4 to the neighbouring *cis* hydroxy group. All the isomers were obtained pure after column chromatography on silicagel. The diols **11** and **13** were successively reacted with dibutyltinoxide and benzyl bromide in the presence of tetrabutyl ammonium bromide[27] to yield the di-*O*-benzoyl mono-*O*-benzyl cyclopentan tetraols **14**, **15** and **16** (Scheme 1).

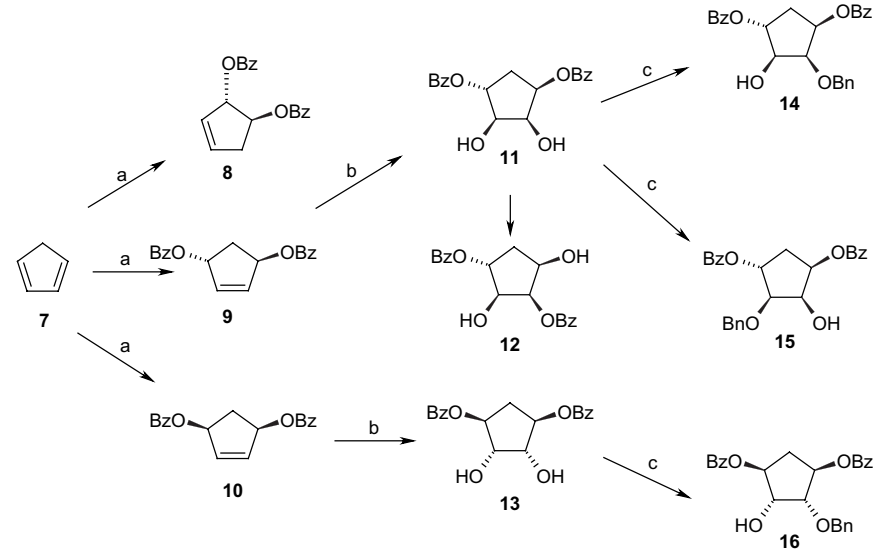
Hydrolysis of the benzoates **14** and **16** by means of sodium hydroxide furnished the triols **17** and **18**. These hydroxyls were phosphorylated using the phosphite method by means of *N*,*N*-diethylamino benzodioxa(1,3,2) phosphepane and tetrazole followed by *m*-chloroperbenzoic acid[28] yielding the protected final products **19** and **20**. Total deprotection was obtained in one step by hydrogenolysis using palladium on charcoal as a catalyst.[21] The expected compounds **5** and **6**, as racemates, were stabilised as cyclohexylammonium salts to prevent phosphate migrations (Scheme 2).

3. Binding properties

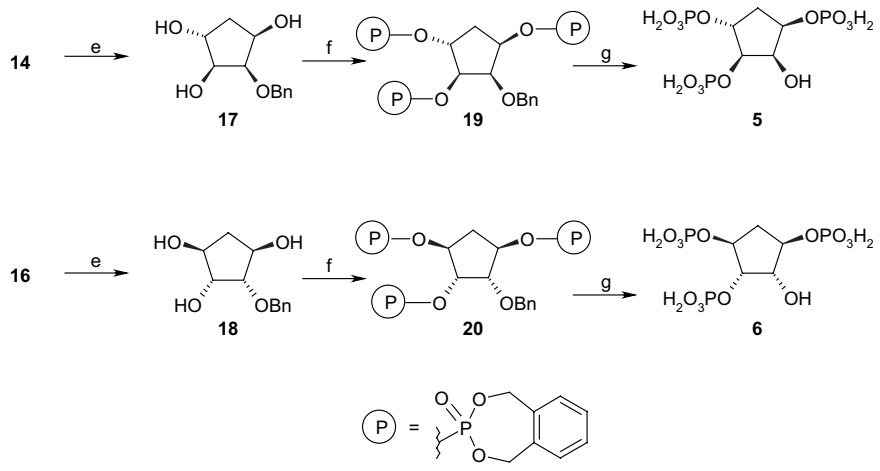
The affinity of the prepared compounds for the Ins(1,4,5)P_3 receptors was measured by binding on bovine adrenal cortex microsomes.[29] The results are reported as K_d values (μM) in Table 1.

4. ^{31}P NMR titrations

As examples, Figure 2 shows the ^{31}P NMR titrations of the tris(phosphate) **5** and the reference compound **1**. As for analogue **4**, the titration curves of the tris(phosphate) **5** appear as a superimposition of an isolated monophasic phosphate for phosphate 4 equivalent to phosphate 1 of Ins(1,4,5)P_3 and a vicinal bis(phosphate) for the phosphates 1 and 2 equivalent to the phosphates 4 and 5 of the parent compound **1**. The epimerisation of hydroxyl 3 does not improve the cooperative intramolecular effects compared to compound **4**. The same behaviour of the phosphates was observed for the tris(phosphate) **6** where the phosphates 1 and 2 were epimerised compared to compound **4** leading to a *cis*



Scheme 1. Reagents and conditions: (a) BzOAg, I_2, dry Et_2O, reflux, yield **8**–**10** 63%; (b) $KMnO_4$, Me_2CO, H_2O, 0 °C, yield **11** 33%, **12** 17%, **13** 66%; (c) Bu_2SnO, Bu_4NBr, CH_3CN, MS 3 Å, BnBr, reflux, yield **14** 27%, **15** 27%, **16** 67%.



Scheme 2. Reagents and conditions: (e) NaOH 1 N, MeOH, THF, rt, yield **17** 41%, **18** 85%; (f) *N*,*N*-diethylamino benzodioxa(1,3,2) phosphepane, *1H*-tetrazole, dry THF, −78 °C, then rt overnight; then −78 °C, *m*-CPBA, CH_2Cl_2, then rt, yield **19** 60%, **20** 55%; (g) H_2, 5 atm, Pd/C 10%, MeOH, CH_2Cl_2, H_2O, AcOH, rt, overnight, then evaporation, H_2O, cyclohexylamine, $(Me)_2CO$, yield **5** 45%, **6** 57%.

Table 1. Binding properties of the synthesised products and reference compounds

N^r	**1**	**4**	**5**	**6**
K_d (μM)	0.025	>25[17]	100	70

system between the phosphate 2 and the hydroxyl group in position 3 (titration curves not shown).

5. Discussion

Among the synthesised cyclopentanic analogues of Ins(1,4,5)P_3, the trisphosphorylated compounds show a significant affinity for the Ins(1,4,5)P_3 receptors. These affinities remained, however, lower than the parent compound and are of the same order of magnitude as the cyclopentanic analogue **4** prepared by Potter et al. One can observe that the epimerisation of the hydroxyl as well as the inversion of the relative configuration of the phosphate maintain about the same level of affinity. The relative orientation of the functional groups does not seem of prime importance in this series. The removal of the hydroxyl in position 5 does not dramatically reduce the affinity confirming Kozikowski's pharmacophore model. In terms of intramolecular cooperative effects, the changes we made on the relative configurations around the cyclopentanic ring do not induce significant modifications on the NMR titrations compared to the observations made for compound **4** and do not correspond to the aspect observed for the parent Ins(1,4,5)P_3 (**1**). Five member ring contracted analogues

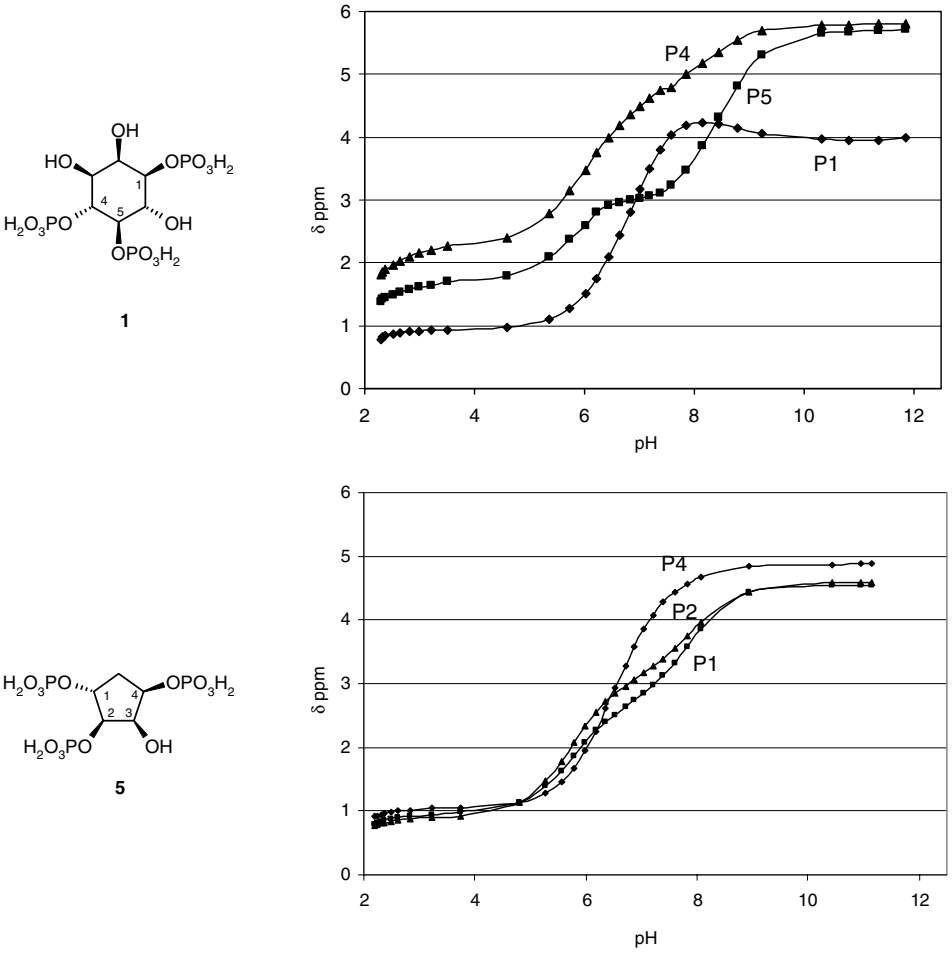


Figure 2. ^{31}P NMR titration analyses of compounds **5** and **1**.

of Ins(1,4,5)P_3 did not seem to lead to highly potent analogues. As the observed affinities were relatively low, it did not seem worth-while to define their agonist or antagonist character.

More work is in progress to understand the SAR. The recently published XR structures of the Ins(1,4,5)P_3 R1 binding domain[30,31] suggest the synthesis of new Ins(1,4,5)P_3 analogues.

6. Experimental part

Melting points were measured on a Mettler PF62 apparatus and are uncorrected. If not specified, NMR spectra were recorded on Bruker Avance 300 spectrometer using the δ scale; the $CHCl_3$ residual signal was fixed at 7.26 ppm. The abbreviations s, d, t, q, qu, m are related to singlet, doublet, triplet, quadruplet, quintuplet and multiplet, respectively. All the new compounds gave satisfactory CHP analyses.

6.1. Dibenzoates 8–10[25]

Sublimated iodine (29.0 g, 0.127 mol) was dropwise added (25 min) to a suspension of silver benzoate[32] (62.0 g, 0.27 mol) in dry ether (500 mL) with stirring. A solution of freshly distilled cyclopentadiene (1 equiv) in dry ether (25 mL) was then slowly added; the exothermic reaction mixture was warming to reflux and the addition rate adjusted to maintain the reflux. After the complete addition, the reflux was maintained by heating for an additional 1 h. The cooled reaction mixture was filtered and the organic layer washed with a saturated aqueous solution of sodium hydrogenocarbonate (2×100 mL), and brine (100 mL) and dried over magnesium sulfate. The solvent volume was reduced to 50 mL and the solution was allowed to slowly crystallise. The crystals were filtered and recrystallised from ethanol giving 8.8 g of *trans*-1,4-cyclohexene dibenzoate **9**. (R_F 0.38 heptane–ethyl acetate 9/1); mp 122 °C; ^{1}H NMR ($CDCl_3$): 2.54 (t, $J = 5.1$, 2H, C*H*$_2$-*5*), 6.16 (td, $J = 5.1$, $J = 0.9$, 2H, *H-1, H-4*), 6.33 (d, $J = 0.9$, 2H, *H-2*, *H-3*), 7.4–8.1 (3m, 10H, (C_6H_5)$_2$). The filtrates were evaporated to dryness and purified by column chromatography on silicagel (300 g) eluted with a heptane–ether gradient starting from 9/1 to 2/1. An oily product possessing the same R_F as the crystals of **9** was isolated but corresponding to the *cis*-1,4-cyclohexene dibenzoate **10**. ^{1}H NMR ($CDCl_3$): 2.11 (dt, $J = 15.5$, $J = 3.9$, 1H, *H-5*β), 3.16 (dt, $J = 14.9$, $J = 7.4$, 1H, *H-5*α), 5.92 (br dd, $J = 7.3$, $J = 4.0$, 2H, *H-1*, *H-4*), 6.33 (br s, 2H, *H-2*, *H-3*), 7.2–8.3 (2m, 10H, (C_6H_5)$_2$). Finally a third isomer was isolated as an oil corresponding to the *trans*-1,2-cylohexene dibenzoate **8**

(R_F 0.28 in heptane–ethyl acetate 9/1).[1] H NMR ($CDCl_3$, 200 MHz): 2.4–2.6 (2m, 1H, *H-5*α), 3.22 (ddq, $J = 18.1$, $J = 7.1$, $J = 1.9$ 1H, *H-5*β), 5.71 (dt, $J = 7.2$, $J = 3.5$, 1H, *H-1*), 6.00 (dq, $J = 5.6$, $J = 1.9$, 1H, *H-4*), 6.1–6.2 (m, 2H, *H-2, H-3*), 7.4–8.2 (3m, 10H, $(C_6H_5)_2$).

6.2. 2β,3β-Dihydroxy cyclohexane 1β,4α-dibenzoate (11) and 1β,3β-dihydroxy cyclohexane 2β,4α-dibenzoate (12)

Cyclopentene dibenzoate **9** (6.16 g, 20 mmol), was added and stirred in a 1 L flask containing 500 mL acetone and cooled to 0 °C. $KMnO_4$ (2.12 g, 20 mmol) dissolved in water (150 mL) was added dropwise. The reaction mixture was kept at 4 °C overnight, filtered and the solid washed with acetone (50 mL). The filtrate was evaporated to dryness and dissolved in ether (200 mL). This organic phase was successively washed with an aqueous K_2CO_3 saturated solution (50 mL) and brine (50 mL) and dried over $MgSO_4$. The crude product obtained after filtration and solvent evaporation was purified on a silicagel column eluted with a gradient starting from heptane–ether 85/15 to heptane–ether–ethyl acetate 5/5/1. Cyclopentene dibenzoate **9** was recovered (1.4 g) followed by the dihydroxylated compound **11** (1.4 g, 26%). R_F heptane–ethyl acetate 1/1: 0.52. ^{1}H NMR ($CDCl_3$): $\overline{3.70}$ (AB part of an ABXY system, $\Delta\delta = 0.32$, $J_{AB} = 15.0$, $J_{AX} = 7.2$, $J_{AY} = 4.7$, $J_{BX} = 6.6$, $J_{BY} = 8.7$, 2H, C*H*$_2$-5), 3.10 (br d, $J = 3.0$, exchangeable with D_2O, 1H, O*H*), 3.72 (br d, $J = 3.0$, exchangeable with D_2O, 1H, O*H*), 4.24 (br q, $J = 3.0$, became t after exchange with D_2O, 1H, C*H*OH), 4.45–4.55 (m, 1H, became t after exchange with D_2O, $J = 3.0$, C*H*OH), 5.34 (dt, $J = 8.7$, $J = 4.4$, 1H, *H-4*), 5.52 (td, $J = 7.2$, $J = 4.4$, 1H, *H-1*), 7.4–8.2 (m, 10H, $(C_6H_5)_2$). The dihydroxylated compound **12** was eluted just after compound **11** (0.8 g, 15%). R_F heptane–ethyl acetate 1/1: 0.44. ^{1}H NMR ($CDCl_3$): $\overline{2.24}$ (AB part of an ABXY system, $\Delta\delta = 0.42$, $J_{AB} = 15.0$, $J_{AX} = 6.2$, $J_{AY} = 5.3$, $J_{BX} = 8.4$, $J_{BY} = 5.0$, 2H, C*H*$_2$-5), 4.40–4.55 (m, 2H, modified after exchange with D_2O, C*H*OH-1 and C*H*OH-3), 5.48 (t, $J = 4.7$, 1H, *H-2*), 5.75 (dt, $J = 8.1$, $J = 5.3$, 1H, *H-4*), 7.3–8.1 (m, 10H, $(C_6H_5)_2$).

6.3. 2β,3β-Dihydroxy cyclohexane 1α,4α-dibenzoate (13)

Same procedure as for **11** starting from the *cis* dibenzoate **10** (11.0 g) and using $KMnO_4$ (3.79 g) led to unreacted starting material (4.0 g) and the expected dihydroxylated derivative **13** (5.3 g, 43%). ^{1}H NMR ($CDCl_3$): $\overline{2.60}$ (AB part of an ABX_2 system, $\Delta\delta = 0.89$, $J_{AB} = 15.3$, $J_{AX} = 3.6$, $J_{BX} = 7.8$, 2H, C*H*$_2$-5), 3.61 (br s, 2H, exchangeable with D_2O, (O*H*)$_2$), 4.39 (d, $J = 1.4$, 2H, *H*-2 and *H*-3), 5.2–5.4 (m, 2H, *H-1*, *H-4*), 7.3–8.2 (m, 10H, $(C_6H_5)_2$).

6.4. 3β-Benzyloxy-2β-hydroxy cyclohexane 1β,4α-dibenzoate (14)

Dibutyltinoxide (10.8 g, 1.2 equiv) and tetrabutylammonium bromide (11.6 g) were added to a solution of the diol **11** (12.5 g, 36.5 mmol) in acetonitrile (500 mL) in a 1 L vessel equipped with a Soxhlet containing 3 Å molecular sieves. The mixture was refluxed for 3 h. Benzyl bromide (29.5 g) was slowly added and the mixture refluxed again for 2 days. Acetonitrile was evaporated and CH_2Cl_2 (300 mL) and $NaHCO_3$ (200 mL saturated aqueous solution) were poured on the residue and the mixture was stirred at room temperature for 1 h. CH_2Cl_2 was recovered and the aqueous solution extracted twice with CH_2Cl_2 (100 mL). The organic layers were dried over $MgSO_4$, filtered and evaporated to dryness giving a crude product, which was purified by column chromatography on silicagel eluted with a mixture of heptane–ether 7/3 leading to the expected monobenzylated derivative **14** (5.2 g, 33%). ^{1}H NMR ($CDCl_3$): $\overline{2.69}$ (AB part of an ABXY system, $\Delta\delta = 0.46$, $J_{AB} = 14.7$, $J_{AX} = 4.2$, $J_{AY} = 7.3$, $J_{BX} = 8.2$, $J_{BY} = 5.9$, 2H, C*H*$_2$-5), 3.20 (d, $J = 4.9$, 1H, exchangeable with D_2O, O*H*), 4.45 (t, $J = 4.5$, 1H, *H*-2), 4.52 (q, $J = 4.5$, became t after exchange with D_2O, 1H, *H*-3), $\overline{4.93}$ (AB, $\Delta\delta = 0.12$, $J_{AB} = 11.6$, 2H, C*H*$_2$–C_6H_5), 5.63 (dt, $J = 8.1$, $J = 3.8$, 1H, *H-4*), 5.85 (br q, $J = 5.4$, 1H, *H*-1) 7.4–8.3 (m, 15H, $(C_6H_5)_3$). The monobenzylated analogue **15** was not obtained pure (4.0 g mixture).

6.5. 2α-Benzyloxy-3-hydroxy cyclohexane 1β,4β-dibenzoate (16)

Same procedure as for **14** starting from the diol **13** (3.5 g) and using dibutyltinoxide (3.02 g), tetrabutylammonium bromide (3.75 g), benzyl bromide (8.3 mL) and acetonitrile (300 mL). Purification by column chromatography on silicagel eluted with a mixture of heptane–ethyl acetate 4/1 gave the expected monobenzylated derivative **16** (3.28 g, 74%). ^{1}H NMR ($CDCl_3$): 1.96 (dt, $J = 15.4$, $J = 3.8$, 1H, *H*-5β), 2.91 (d, $J = 5.6$, 1H, became s after exchange with D_2O, 1H, *OH*), 3.04 (dt, $J = 15.5$, $J = 7.9$, 1H, *H*-5α), 4.23 (dd, $J = 4.3$, $J = 4.1$, 1H, *H*-2), 4.44 (q, $J = 5.3$, became t after exchange with D_2O, 1H, *H*-3), $\overline{4.81}$ (AB system, $\Delta\delta = 0.12$, $J_{AB} = 11.7$, 2H, C*H*$_2$–C_6H_5), 5.30 (dt, $J = 7.5$, $J = 4.1$, 1H, *H*-4), 5.50 (dt, $J = 7.5$, $J = 3.4$, 1H, *H*-1), 7.2–8.1 (m, 15H, $(C_6H_5)_3$).

6.6. 2β-Benzyloxy-1β,3β,4α-trihydroxy cyclohexane (17)

Dibenzoate **14** (1.2 g) dissolved in methanol (25 mL) and THF (25 mL) was stirred overnight at room temperature in the presence of NaOH (20 mL, 1 N). The pH was then adjusted to 6 by addition of HCl (1 N) and the mixture was evaporated to dryness. The crude product was purified on a silicagel column eluted with a mixture of ethyl acetate–methanol 9/1 leading to the expected triol **17** (240 mg, 47%). ^{1}H NMR (200 MHz, $CDCl_3$): $\overline{2.02}$ (AB part of an ABXY system, $\Delta\delta = 0.43$, $J_{AB} = 14.7$, $J_{AX} = J_{AY} = 5.6$, $J_{BX} = 7.8$, $J_{BY} = 2.7$, 2H, C*H*$_2$-5), 3.38 (br s, 3H, exchangeable with D_2O, (*OH*)$_3$), 3.97 (br s, 2H,), 4.23 (dd, $J = 4.3$, $J = 4.1$, 1H, *H*-2), 4.32 (br s, 2H,), 4.72 (s, 2H, C*H*$_2$–C_6H_5), 7.2–7.7 (m, 5H, C_6H_5).

6.7. 2α-Benzyloxy-1β,3α,4β-trihydroxy cyclohexane (18)

Same procedure as for **17** starting from dibenzoate **16** (1.6 g) leading to the triol **18** (690 mg, 84%). Mp 114.5 °C (dec). ^{1}H NMR (200 MHz, $CDCl_3$): 1.59 (dt, 1H, *H*-5), 2.48, dt, 1H, *H*-5), 2.64 (br s 3H, exchangeable with D_2O, $(OH)_3$), 3.9–4.3 (m, 4H, *H*-1, *H*-2, *H*-3, *H*-4), 4.68 (AB system, $\Delta\delta = 0.06$, $J_{AB} = 11.5$, 2H, CH_2–C_6H_5-2), 7.2–7.5 (m, 5H, C_6H_5).

6.8. 2β-Benzyloxy-cyclohexyl 1β,3β,4α-tris(xylyl-phosphate) (19)

The triol **17** (240 mg, 1.08 mmol) was dissolved in dry THF and stirred under inert argon atmosphere and cooled to −78 °C. 1*H*-tetrazole (925 mg, 13.2 mmol) was added followed by *N*,*N*-diethylamino benzodioxa (1,3,2) phosphepane (3.20 g, 6.6 mmol). The reaction mixture was warmed up and kept at room temperature overnight. Then the vessel was cooled again to −78 °C and dry *m*-CPBA (3.25 g) dissolved in dry CH_2Cl_2 (20 mL) was added dropwise. After warming to room temperature, the mixture was stirred for an additional 30 min and the solvents cautiously evaporated in vacuo. The residue was dissolved again in CH_2Cl_2 (300 mL), which was successively washed with $Na_2S_2O_3$ (2 × 50 mL aqueous saturated solution) and $NaHCO_3$ (100 mL aqueous saturated solution) and finally dried over $MgSO_4$. The crude product obtained after concentration in vacuo was purified on a silicagel column eluted with a mixture of CH_2Cl_2–CH_3OH 99-1 to 80/20, giving the expected tris(phosphate) **19** (502 mg, 60%). Mp 80 °C. Analyses calculated for $C_{36}H_{37}O_{13}P_3$; $1/2H_2O$ (770.61): C, 55.47; H, 4.91; P, 11.92; found: C, 55.24; H, 5.02; P, 11.46. ^{1}H NMR (200 MHz, $CDCl_3$): 2.60 (AB part of an ABXY system, $\Delta\delta = 0.49$, $J_{AB} = 15.0$, $J_{AX} = 7.2$, $J_{AY} = 4.1$, 2H, CH_2-5), 4.28 (t, $J = 4.7$, 1H, C*H*–OBn), 4.7–5.5 (m, 17H, $(CH_2C_6H_5)_7 + (CH\text{–}OP)_3$), 7.0–7.6 (m, 17H, $C_6H_5 + (C_6H_4)_3$).

6.9. 3α-Benzyloxy-cyclohexyl 1β,2β4β-tris(xylyl-phosphate) (20)

Same procedure as for **19** starting from 2α-benzyloxy-1β,3α,4β-trihydroxy cyclohexane (**18**) (630 mg, 2.81 mmol) and using tetrazole (1.62 g, 8 equiv), *N*,*N*-diethylamino benzodioxa(1,3,2) phosphepane (5.60 g, 6 equiv), *m*-CPBA (5.7 g), produced tris(phosphate) **20** (1.20 g) after purification on a silicagel column eluted with a mixture of heptane–ethyl acetate 6/4. Mp 61 °C. ^{1}H NMR ($CDCl_3$): 2.21 (dt, $J = 15.4$, $J = 3.0$, 1H, *H*-5β), 2.92 (dt, $J = 15.6$, $J = 7.8$, 1H, *H*-5α), 4.30 (t, $J = 3.1$, 1H, *H*-3), 4.71 (AB system, $\Delta\delta = 0.03$, $J_{AB} = 11.6$, 2H, CH_2–C_6H_5), 4.8–5.4 (m, 15H, $(CH_2C_6H_4CH_2)_3$, *H*-1, *H*-2 and *H*-4), 7.1–7.4 (m, 17H, C_6H_5 and $(C_6H_4)_3$).

6.10. 3β-Hydroxy-cyclohexyl 1α,2β,4β-tris(phosphate) (5)

The totally protected tris(phosphate) **19** (200 mg, 0.26 mmol) was dissolved in a mixture of CH_3OH (16 mL), H_2O (8 mL), CH_2Cl_2 (8 mL) and CH_3CO_2H (1 mL). Pd/C 10% (200 mg) was added and the mixture was hydrogenolysed under 5 atm in a Parr shaker for 10 h. After filtration, the solvents were evaporated in vacuo, the residue redissolved in pure water (5 mL), and cooled to 0 °C. Cyclohexylamine (2 mL) was added and the mixture was evaporated again to dryness in vacuo. The residue was dissolved in pure water (1 mL) and dropped in dry acetone (150 mL). The precipitate was filtered and redissolved in water (1 mL) and precipitated again in acetone (150 mL). The beige powder was filtered and dried in vacuo leading to the expected product as its pentacyclohexylammonuim salt (113 mg, 50%). Mp (dec). Analyses calculated for $(C_5H_7O_{13}P_3)^{6-}$; $4C_6H_{14}N^+$; $2Na^+$ (814.74): C, 42.75; H, 7.7; P, 11.40; N, 6.72; found C, 42.33; H, 8.19; P, 10.78; N, 6.64. ^{1}H NMR (200 MHz, D_2O, CD_3OD 1/1 + COSY): 0.9–2.0 (6m, 51H, C*H*-5, 25 CH_2 cyclohexyl), 2.1–2.3 (m, 1H, *H*-5), 2.9–3.0 (m, 5H, $(NH_2CH)_5$), 4.09 (t, $J = 4.0$, 1H, *H*-3), 4.26 (dt, $J = 9.2$, $J = 4.7$, became t after $\{^{31}P\}$, 1H, *H*-2), 4.42–4.51 (m, became td after $\{^{31}P\}$, $J = 7.4$, $J = 3.9$, 1H, *H*-4), 4.51–4.61 (m, became td after $\{^{31}P\}$, $J = 9.2$, $J = 5.0$, 1H, *H*-1); ^{13}C NMR (75.48 MHz, D_2O, CD_3OD 1/1 + HSQC): 24.15, 24.63, 30.74, 50.55 (cyclohexylammonium), 36.88 (*C*-5), 72.68 (*C*-3), 73.17 (*C*-2), 78.30 (*C*-4), 80.40 (*C*-1). $\{^1H\}$ ^{31}P NMR (80.96 MHz, H_2O, D_2O 95/5, KCl 2 M, pH 7.2): 2.97, 3.27, 4.08.

6.11. 3α-Hydroxy-cyclohexyl 1β,2α,4β-tris(phosphate) (6)

Same procedure as for **5** starting from the protected compound **26** (710 mg, 1.00 mmol) gave tris(phosphate) **7** as its hexacyclohexylammonium salt (546 mg, 61%). Mp (dec). Analyses calculated for $(C_5H_7O_{13}P_3)^{6-}$; $6C_6H_{14}N^+$, $2H_2O$: C, 48.99; H, 9.53; N, 8.36; P, 9.25; found: C, 48.68; H, 9.83; N, 7.99; P, 8.83. ^{1}H NMR (D_2O + COSY): 0.9–2.0 (4m, 61H containing at 1.63 C*H*-5β as the A part of an ABXY system + 30 CH_2 cyclohexyl), 2.97 (dt, $J = 15.0$, $J = 6.8$, 1H, *H*-5α), 2.9–3.0 (m, 6H, $(NH_2CH)_5$), 4.03 (t, $J = 4.5$, 1H, *H*-3), 4.25 (dt, $J = 7.4$, $J = 4.7$, became dd after $\{^{31}P\}$, 1H, *H*-4), 4.35–4.50 (m, simplified after $\{^{31}P\}$, 2H, *H*-2, *H*-1); $\{^1H\}$ ^{31}P NMR (80.96 MHz, H_2O, D_2O 95/5, KCl 2 M, pH 6.8): 2.54, 2.84, 3.23.

6.12. ^{31}P NMR titrations

The acid base properties of the synthesised products were studied using cyclohexyl phosphate as internal pH sensor. Thus, the tris(phosphate) (7.12 mg) and cyclohexyl phosphate (1.5 equiv) cyclohexyl ammonium salts were eluted through an Amberlist IRN 77 column in its acidic form to regenerate the free acids as previously reported.[15] The lyophilised acids were dissolved in 0.5 mL D_2O/H_2O (5/95) 0.2 M in KCl. The initial pH was measured and the solution introduced in a 5 mm NMR tube at 26 °C. Between each $\{^1H\}^{31}$P spectrum acquisition, 10 μL NaOH (0.2 N) were added. The internal pH was obtained using the cyclohexyl phosphate chemical shift, which was compared to this of a

previous titration of this compound. The final pH was also measured using a classical pH electrode.

6.13. Binding assay

Bovine adrenal cortices (dissected free of medullary tissue) were homogenised with eight strokes of a Dounce homogeniser (loose pestle) in a medium containing 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 25 mM Tris–HCl pH 7.2, 5 mM KH_2PO_4, 1 mM dithiothreitol and 2 mM EGTA. After stirring for 5 min and centrifugation at 500*g* for 15 min, the supernatant was centrifuged at 35,000*g* for 20 min. The pellet was resuspended in the medium without EGTA, supplemented with glycerol (1.4% w/v), at a concentration of 20–30 mg of protein/mL. The microsomes were stored at −70 °C. This microsomal preparation has been successfully used since 1987.[29]

Bovine adrenal microsomes were incubated for 30 min at 0 °C in a medium containing 25 mM Tris/HCl buffered at pH 8.5, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4 and 1 mM EDTA in a final volume of 500 μL with appropriate concentration of [^{3}H]-Ins(1,4,5)P_3 (0.9 nM), Ins(1,4,5)P_3 and Ins(1,4,5)P_3 derivatives. Nonspecific binding was determined in the presence of 1 μM Ins(1,4,5)P_3. Incubations were terminated by centrifugation at 15,000*g* for 5 min at 0 °C. The receptor-bound radioactivity was analysed by liquid scintillation spectrometry.

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References and notes

1. Streb, H.; Irvine, R. F.; Berridge, M. J.; Schultz, I. *Nature* **1983**, *312*, 375–376.
2. Berridge, M. J.; Irvine, R. F. *Nature* **1984**, *312*, 315–321.
3. Berridge, M. J. *Nature* **1993**, *361*, 315–325.
4. Billington, D. C. *Chem. Soc. Rev.* **1989**, *18*, 83–122.
5. Billington, D. C. *The Inositol Phosphates*; VCH: Weinheim, 1993. ISBN 3-527-28152-5; p 153.
6. Potter, B. V. L.; Lampe, D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1933–1972.
7. Kozikowski, A. P.; Ognyanov, V. I.; Fauq, A. H.; Nahorski, S. R.; Wilcox, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 4429–4434.
8. Bonis, D.; Sezan, A.; Mauduit, P.; Cleophax, J.; Gero, S. D.; Rossignol, B. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 894–900.
9. Takahashi, M.; Kagasaki, T.; Hosoya, T.; Takahashi, S. *J. Antibiot. (Tokyo)* **1993**, *46*, 1643–1647.
10. Takahashi, M.; Tanzawa, K.; Takahashi, S. *J. Biol. Chem.* **1994**, *269*, 369–372.
11. Correa, V.; Riley, A. M.; Shuto, S.; Horne, G.; Nerou, E. P.; Marwood, R. D.; Potter, B. V. L.; Taylor, C. W. *Mol. Pharmacol.* **2001**, *59*, 1206–1215.
12. Murphy, C. T.; Riley, A. M.; Lindley, D. J.; Jenkins, D. J.; Westwick, J.; Potter, B. V. L. *Mol. Pharmacol.* **1997**, *52*, 741–748.
13. Roussel, F.; Moitessier, N.; Hilly, M.; Chretien, F.; Mauger, J. P.; Chapleur, Y. *Bioorg. Med. Chem.* **2002**, *10*, 759–768.
14. Beecroft, M. D.; Marchant, J. S.; Riley, A. M.; van Straten, N. C. R.; van der Marel, G. A.; van Boom, J. H.; Potter, B. V. L.; Taylor, C. W. *Mol. Pharmacol.* **1999**, *1*, 109–117.
15. Jenkins, D. J.; Potter, B. V. L. *J. Chem. Soc., Chem. Commun.* **1995**, 1169–1170.
16. Jenkins, D. J.; Potter, B. V. L. *Carbohydr. Res.* **1996**, *287*, 169–182.
17. Jenkins, D. J.; Potter, B. V. L. *J. Chem. Soc., Perkin Trans. 1* **1998**, 41–49.
18. Schmitt, L.; Schlewer, G.; Spiess, B. *Biochim. Biophys. Acta* **1991**, 139–140.
19. Guédat, P.; Poitras, M.; Spiess, B.; Guillemette, G.; Schlewer, G. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1175–1178.
20. Schmitt, L.; Bortmann, P.; Schlewer, G.; Spiess, B. *J. Chem. Soc., Perkin Trans. 1* **1993**, 2257–2263.
21. Ballereau, S.; Guedat, P.; Poirier, S. N.; Guillemette, G.; Spiess, B.; Schlewer, G. *J. Med. Chem.* **1999**, *42*, 4824–4835.
22. Felemez, M.; Ballereau, S.; Schlewer, G.; Spiess, B. *New J. Chem.* **2000**, *24*, 631–638.
23. Felemez, M.; Bernard, P.; Schlewer, G.; Spiess, B. *J. Am. Chem. Soc.* **2000**, *122*, 3156–3165.
24. Felemez, M.; Schlewer, G.; Jenkins, D. J.; Correa, V.; Taylor, C. W.; Potter, B. V. L.; Spiess, B. *Carbohydr. Res.* **1999**, 95–101.
25. Gaoni, Y. *Bull. Soc. Chim. Fr.* **1950**, 705–710.
26. Sable, H. Z.; Anderson, T.; Tolbert, B.; Posternak, T. *Helv. Chim. Acta* **1963**, 1157–1165.
27. Nagashima, N.; Ohno, M. *Chem. Lett.* **1987**, 141–144.
28. Perich, J. W.; Johns, R. B. *Tetrahedron Lett.* **1987**, *28*, 101–102.
29. Guillemette, G.; Balla, T.; Baukal, A. J.; Spat, A.; Catt, K. J. *J. Biol. Chem.* **1987**, *262*, 1010–1015.
30. Serysheva, I. I.; Bare, D. J.; Ludtke, S. J.; Kettlun, C. S.; Chiu, W.; Mignery, A. *J. Biol. Chem.* **2003**, *278*, 21319–21322.
31. Bosanac, I.; Alattla, J. R.; Mal, T. K.; Chan, J.; Talarico, S.; Tong, F. K.; Tong, K. I.; Yoshlkawa, F.; Furulchl, T.; Iwal, M.; Michlkawa, T.; Mikoshiba, K.; Ikura, M. *Nature* **2002**, *420*, 696–700.
32. Neumann, M. S.; Beal, P. F. *J. Am. Chem. Soc.* **1950**, *72*, 5163–5165.