^{13}C Labelling and electrospray mass spectrometry reveal a *de novo* route for inositol biosynthesis in *Leishmania donovani* parasite

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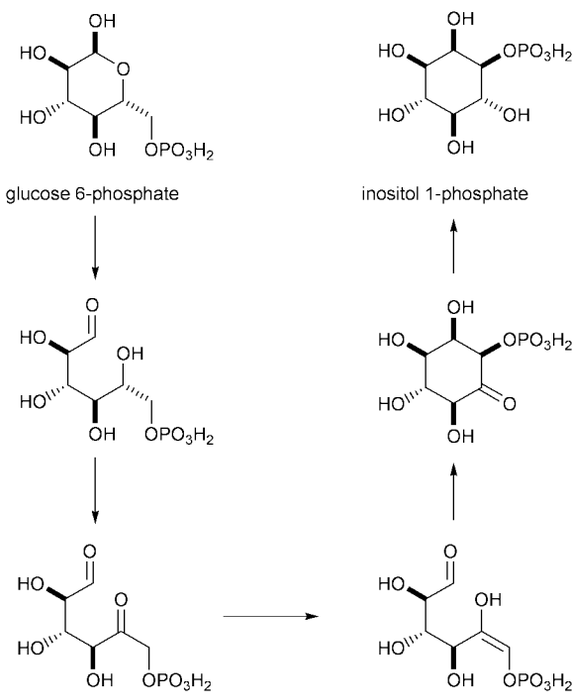
In this paper we provide evidence for the presence of a *de novo* route for *myo*-inositol biosynthesis in the protozoan parasite *Leishmania donovani*, by the application of ^{13}C labelling and electrospray ionisation mass spectrometry (ESMS). For this *myo*-[1-^{13}C]inositol is prepared from D-[6-^{13}C]glucose and biosynthetically incorporated in the parasite promastigote cell culture. Biosynthetic phosphatidylinositol (PI) and its hydrolysis products glycero-PI and inositol are analysed by ESMS and the isotopomeric ratio determined. The incorporation experiments show substantial isotopic dilution, indicating the presence of *myo*-inositol 1-phosphate synthase (MIP synthase) enzyme in the parasite; this is further confirmed by incorporation of D-[6-^{13}C]glucose in the parasite phosphatidylinositol.

Introduction

The identification of major cell surface **g**lycosyl**p**hosphatidyl-**i**nositol (GPI) molecules[1] from the protozoan parasites (*Leishmania*, *Trypanosome* and malaria) and their immunological role[2] in the parasite infectivity and survival has coincided with the discovery[3] of an important transmembrane signal-tranduction pathway mediated by phosphatidylinositols and inositol phosphates. This has provided new opportunities to study host–parasite relationships and design strategies to combat major tropical diseases due to these parasites. *Leishmania donovani,* causing visceral leishmaniasis (kala azar), and related species express two major type of GPI structures, namely lipophosphoglycan (LPG) and glycosylated inositol phospholipids (GIPLs), in very high copy numbers (more than 10^7 molecules cell^{-1}) on their cell surface which help the parasite to infect and proliferate within human macrophages. This has generated major interest in their biosynthesis[1] and chemical synthesis.[4] *Leishmania* parasites should require huge supplies of inositol for synthesis of phosphatidylinositols (required for signalling) and GPIs (for membrane anchoring of protein and carbohydrate antigens, receptors and adhesion molecules). Therefore the question of whether the parasite sequesters inositol from its hosts or it possesses machinery for inositol biosynthesis is of interest as it may provide new targets for chemotherapeutic intervention.

The biosynthesis of *myo*-inositol in yeast and bacteria is mediated by the enzyme *myo*-inositol 1-phosphate synthase (MIP synthase) which has been isolated[5] and cloned[6] in yeast. MIP synthase is a unique enzyme catalysing (Scheme 1) oxidation, enolisation, intramolecular aldol-condensation and carbonyl-reduction steps involved in the transformation of glucose 6-phosphate to inositol 1-phosphate. The precise mechanism of MIP synthase has not been elucidated but 5-keto-glucose 6-phosphate and *myo*-2-inosose 1-phosphate have been proposed[7] as intermediates, and the latter was found[8] to be a competitive inhibitor of the enzyme. Interestingly, in humans MIP synthase is produced[9] only in the brain and testes and is not expressed in cells infected by *Leishmania* and malaria parasites. In spite of a major metabolic requirement of inositol for signalling and GPI biosynthesis, there has been no report of the source of inositol or presence of MIP synthase activity in the *Leishmania*, *Trypanosome* and malaria parasites. In continuation of our work[10–12] on *L. donovani* phospholipids and GPI molecules, we decided to explore the biosynthesis of inositol in this parasite. In this paper we describe the use of stable isotope [^{13}C] labelling and electrospray ionisation mass spectrometry (ESMS) to address the question of *myo*-inositol biosynthesis in *Leishmania* parasites. The approach using ^{13}C-labelled inositol and glucose as precursors to study inositol biosynthesis in the parasite was adopted for the following reasons: (a) in a cell culture system parasites take up inositol from the medium (radiolabelling studies) to incorporate in PI/GPI molecules, (b) if the parasite was also making its own inositol (MIP synthase) from glucose, the ^{13}C enrichment level would become diluted in PI/GPI products. Negative-ion ESMS was found particularly suitable for the analysis of parasitic phospholipids, phosphatidylinositol (PI) and deacylated glycero-PI, which gave promin-



Scheme 1 Proposed mechanism of MIP synthase.

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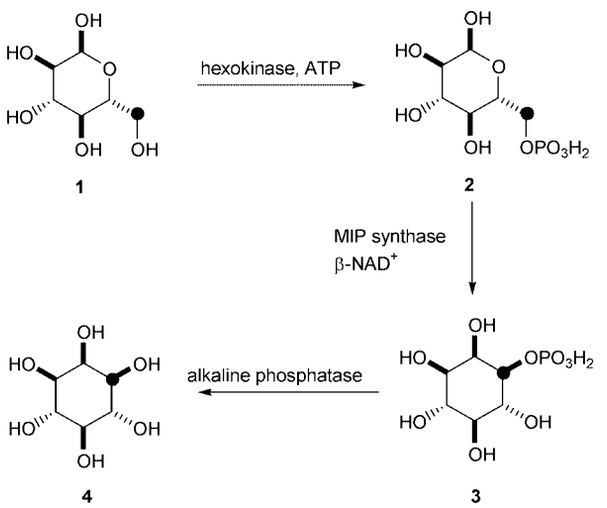
ent $[M - H]^-$ ions. The incorporation of *myo*-[1-^{13}C]inositol and D-[6-^{13}C]glucose in the parasite, and isotope dilution/enrichment measurement of biosynthetic PI and inositol by ESMS, led to identification of both *de novo* inositol biosynthesis and the presence of MIP synthase in the parasite.

Results and discussion

Two routes for the enantioselective synthesis of 1D-*myo*-[1-^{13}C]-inositol were devised using D-[6-^{13}C]glucose as a suitable starting material available in stable-isotope-labelled forms. In the first, enzymic, approach (Scheme 2), we designed an *in-vitro*



Scheme 2 Enzymic synthesis of *myo*-[1-^{13}C]inositol using MIP synthase from *S. cerevisae opi 1δ* mutant (● = ^{13}C).

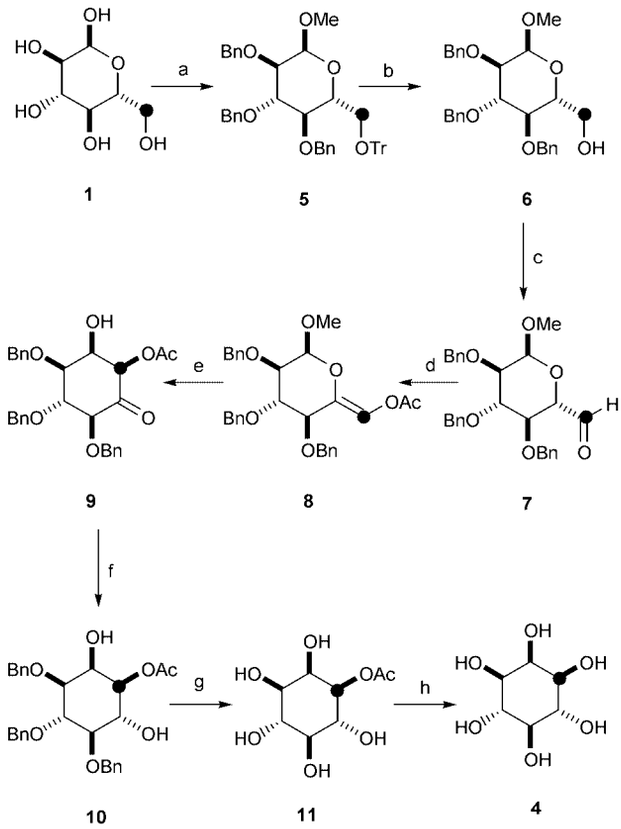
synthesis using a mutant strain of yeast (*Saccharomyces cerevisiae*) over-expressing *myo*-inositol 1-phosphate synthase. The second, chemical, route (Scheme 3) was based on the Ferrier reaction [13] which involves intramolecular aldol-type carbocyclisation of a suitably protected glucose 5-enol acetate.[14]

Enzymic synthesis of *myo*-inositol

In the first step D-glucose **1** was treated with hexokinase [15] (Type VI from baker's yeast) and ATP (Scheme 2) to give D-glucose 6-phosphate **2** in 81% yield. The next step involved transformation of **2** to *myo*-inositol 1-phosphate **3** for which partially purified MIP synthase from a genetic mutant of yeast (*S. cerevisiae*, ATCC 74033, genotype *MATa*, *opi 1δ*, with MIP synthase subunit being constitutively over-expressed) [16] grown in YEPD medium was used. In a typical incubation, 57 units of enzyme and β-NAD^+ were used for 0.306 mM glucose 6-phosphate substrate, and purification from anion- (AG1-X8) followed by cation-exchange (Dowex HCR W2) chromatography yielded 17.2 mg of *myo*-inositol 1-phosphate (21.7%). Finally, dephosphorylation of **3** by alkaline phosphatase (*E. coli*), followed by ion-exchange purification gave *myo*-inositol **4** in 63% yield (6.7 mg).

Chemical synthesis of *myo*-inositol

In an alternative strategy (Scheme 3), D-glucose was first converted to methyl 2,3,4-tri-*O*-benzyl-6-*O*-(triphenylmethyl)-D-glucopyranoside **5** by a published method [17] followed by detritylation to give methyl 2,3,4-tri-*O*-benzyl-D-glucopyranoside **6**. The next three steps included Swern oxidation of primary hydroxy group of **6** to aldehyde **7**, preparation of the enol acetate **8** and Ferrier rearrangement to give the corresponding inosose **9**. Stereoselective hydroxy-directed hydride reduction [14,18] of **9** with sodium triacetoxyborohydride led to 1-*O*-acetyl-3,4,5-tri-*O*-benzyl-*myo*-inositol **10** in good yield,



Scheme 3 Synthesis of 1D-*myo*-[1-^{13}C]inositol (● = ^{13}C). *Reagents and conditions*: (a) (i) MeOH, Dowex H^+ resin, (ii) trityl chloride, pyridine, DMAP, (iii) BnBr, NaH, DMF; (b) *p*-TsOH, pH 4; (c) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C to rt; (d) Ac_2O, K_2CO_3, CH_3CN, 80 °C; (e) $Hg(OAc)_2$, acetone–water, rt, 45 min; then saturated aq. NaCl, rt, 24 h; (f) $NaBH(OAc)_3$, AcOH, CH_3CN; (g) 10% Pd/C, hydrogen, 10 atm; (h) NaOMe, MeOH.

and C^2-OH axial stereochemistry was confirmed by detailed analysis of the vicinal coupling constants and NOE difference experiments. Compound **10** was debenzylated (10% Pd/carbon at 10 atm hydrogen) to 1D-1-*O*-acetyl-*myo*-inositol **11** which on deacetylation (NaOMe, MeOH) and purification by Amberlite IR-120 (H^+) gave the desired D-*myo*-inositol **4**.

Synthesis of *myo*-[1-^{13}C]inositol

For preparation of labelled *myo*-[1-^{13}C]inositol, both the enzymic (Scheme 2) and chemical (Scheme 3) approaches were applied using D-[6-^{13}C]glucose **1** (99 atom-% enrichment) as the starting material and sufficient amounts (22.5 mg) of *myo*-[1-^{13}C]inositol were obtained from 200 mg of labelled glucose. The isotopomeric ratio was determined by negative-ion ESMS (Fig. 1, panel A1) and ^{13}C NMR data. For larger scale synthesis, we found the chemical route more convenient as it involved less cumbersome purification steps. The overall yield for the glucose-to-inositol synthesis by the chemical route (10 steps) was 11.25%, and for the enzymic route (three steps) was 9.9%. This is the first reported preparation of ^{13}C-labelled *myo*-inositol which would be of potential use to phosphoinositide signal-transduction studies.

Establishment of ESMS conditions for isotopomer analysis

For monitoring of the incorporation of *myo*-[1-^{13}C]inositol in the parasitic cell-surface inositides, a suitable experimental methodology was first established. This included (a) parasite promastigote culture, (b) establishment of biosynthetic viability by radioactive labelling and identification of PI/GPI molecules, (c) deacylation of fatty esters of parasite diacyl-PI to glycero-PI by NH_4OH; this was done to facilitate separation of water-

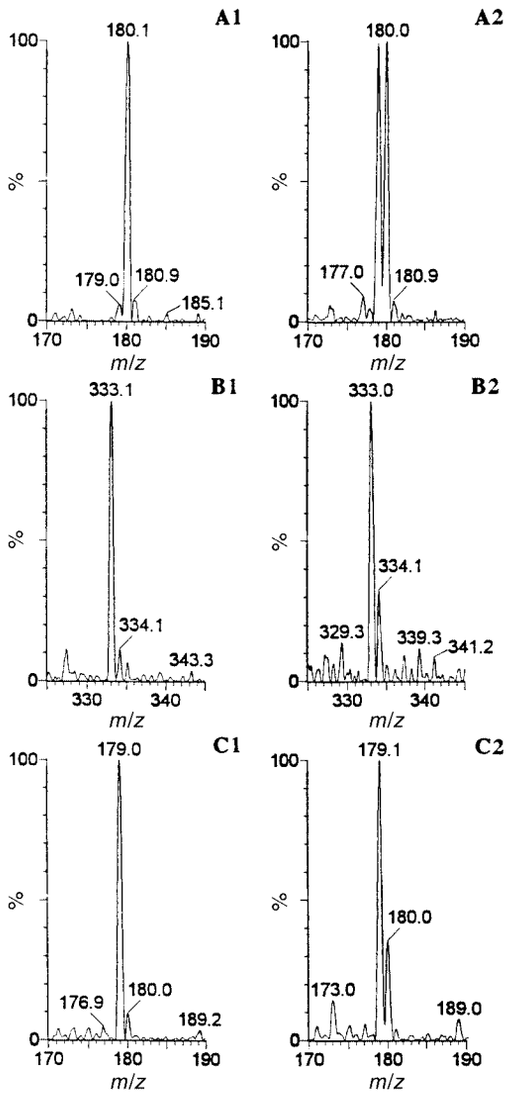


Fig. 1 ESMS (negative-ion) data from experiment on biosynthetic incorporation of *myo*-[1-^{13}C]inositol in *Leishmania donovani*: (**A1**) ESMS of synthetic *myo*-[1-^{13}C]inositol prepared from D-[6-^{13}C]glucose (99 atom-% ^{13}C); (**A2**) ^{13}C enrichment level of inositol present in the culture medium at the start of incubation; (**B1**) glycero-PI from control experiment; (**B2**) glycero-PI from labelling experiment; (**C1**) inositol obtained by hydrolysis of glycero-PI from control experiment; (**C2**) inositol obtained by hydrolysis of glycero-PI from labelling experiment.

soluble glycero-PI from other phospholipids, and GPIs which in *Leishmania* parasites contain a 1-*O*-alkyl-2-*O*-acyl-PI moiety, (d) hydrolysis of the phosphodiester group of glycero-PI to obtain biosynthetic inositol. In our recent study on *Leishmania* cell-surface phospholipids, it was observed that glycero-PI derived from the parasite gave a clean and prominent molecular ion *m/z* 333 $[M - H]^-$ under negative-ion ESMS conditions, suitable for determination of the isotopomeric ratios of the biosynthetic product. Therefore it was decided to exploit this observation for the present ^{13}C-labelling study.

The parasite PI/GPI fraction was digested with 4 M NH_4OH at room temp. and, after purification, ESMS showed two prominent quasimolecular ions $[M - H]^-$ at *m/z* 333.1 and 242.1 for glycero-PI and glycero-PC, respectively. To confirm the identity of these compounds, three samples (PI, PC and soybean lecithin containing both PI and PC) were digested separately with 4 M NH_4OH and analysed by ESMS which showed *m/z* 333.1 for glycero-PI and *m/z* 242.1 for the glycero-PC, and for the lecithin sample both these ions were seen; the structures were also confirmed by ^{1}H and ^{31}P NMR spectroscopy. Further digestion of glycero-PI in 10% NH_4OH at 150 °C for 18 h followed by purification led to the free *myo*-inositol, showing a single peak at *m/z* 179 $[M - H]^-$ in ESMS. This was in view of the report [19] that 10% NH_4OH hydrolysis at 150 °C released free inositol from PI/glycero-PI by hydrolysis of phosphodiester, and not from the glycosylated-PI molecules, which allowed efficient separation of PI and GPI.

Having established release and identification of glycero-PI and inositol from the parasite and the ESMS conditions, a calibration curve for quantitative measurement of the isotopic ratio of ^{12}C:^{13}C in biosynthetic products was prepared. For this, [6-^{13}C]glucose **1** was diluted with unlabelled glucose to give 1, 2, 5, 10, 20, 30, 40, and 50 atom-% and these samples were analyzed by ESMS and the area under the peaks at *m/z* 179 (unlabelled) and 180 (^{13}C-labelled) were measured and plotted in a calibration curve that was used throughout the study to estimate isotope dilution or enrichment during biosynthesis.

Biosynthetic incorporation of *myo*-[1-^{13}C]inositol in *Leishmania* parasite

L. donovani parasite promastigotes (DD8 strain) were first starved in inositol-free Medium-199 with fat-free BSA for 24 h; this was done to deplete free inositol in the cytosolic pool of the parasite, and to increase the level of expression of putative MIP synthase. After starving, parasites were harvested, washed, and used for incubation in normal unlabelled (control) and *myo*-[1-^{13}C]inositol-rich (labelling) media. Initially [1-^{13}C]inositol (1.4 mg, 99 atom-%) was diluted with unlabelled inositol (0.6 mg) to bring the ^{13}C level to 70 atom-%; this labelled precursor (2 mg) was added to the biosynthetic incubation. At the beginning (time $t = 0$) of the incubation, small aliquots from the parasite cultures (*labelling* as well as *control*) were taken for the determination of ^{13}C atom-% of the precursor. From these culture aliquots, inositol was isolated by extraction of inositides, hydrolysis and preparative TLC (PLC). The ESMS of inositol from the *labelling* sample showed that the ^{13}C atom-% of precursor inositol was reduced to 50% (Fig. 1, panel A2) due to minor amounts of unlabelled inositol present in the commercial medium used for the parasite culture. It is important to mention here that instead of analyzing the culture directly by ESMS to determine the initial ^{13}C label of precursor inositol, TLC purification was necessary to eliminate the contribution of glucose which was present in the medium and also gave an $[M - H]^-$ peak at *m/z* 179. Inositol and glucose were clearly resolved (R_f of inositol 0.15, and glucose 0.34).

After incubation of promastigotes for 24 h for both the control and biosynthetic labelling experiments, the parasites were processed to isolate PI, glycero-PI and inositol as described in the earlier section. The ESMS data of the control and labelling experiments are listed in Table 1. The ratios of the abundances of the pseudomolecular ions, *i.e.* $[M - H]^-$ or P at *m/z* 333 to P + 1 at 334 and P + 2 at 335 for control glycero-PI, and $[M - H]^-$ or P at *m/z* 179 to P + 1 at 180 and P + 2 at 181 for control *myo*-inositol, were very close to the theoretical values. ESMS of the glycero-PI from labelling experiment showed (Fig. 1) that the ^{13}C label from precursor *myo*-[1-^{13}C]inositol was incorporated as expected into glycero-PI and the relative abundance of peak at *m/z* 334 (P + 1) was found to be 21% more in glycero-PI from the labelling experiment (Fig. 1, panel B2) than that from the control experiment (Fig. 1, panel B1). More interestingly when this ^{13}C ratio in glycero-PI from the labelling experiment (Fig. 1, panel B2) was compared with the ^{13}C atoms (50%) present at the beginning of incubation (Fig. 1, panel A2), it was clear that there was substantial dilution of the label (from 50 to 21%) during incorporation of *myo*-[1-^{13}C]-inositol. This could have happened only when a *de novo* biosynthesis of inositol was also in operation in the parasite, converting glucose to inositol (MIP synthase activity) leading to dilution of the isotope atom-%.

To confirm this observation, the glycero-PI from control and labelling experiments were further hydrolysed with 10%

Table 1 Electrospray mass spectrometry experimental results

	glycero-PI ($P/P + 1/P + 2$) $P = 333$		*myo*-Inositol ($P/P + 1/P + 2$) $P = 179$	
	Control	Biosynthetic	Control	Biosynthetic
Peak intensity proportions from labelling with [1-^{13}C]inositol	100:8:2	100:35:4	100:11:2	100:26:4
Peak intensity proportions from labelling with [6-^{13}C]glucose	100:8:2	100:43:10	100:11:2	100:48:11

NH_4OH at 150 °C in sealed tubes and purified (PLC) to obtain hydrolysed free inositol. The ESMS of the samples showed the relative abundance of peak at *m/z* 180 to be 13% more in inositol obtained by hydrolysis of glycero-PI from labelling experiment (Fig. 1, panel C2) than that from the control experiment (Fig. 1, panel C1). The starting ratio of peaks at *m/z*, 179/180 (1:1, Fig. 1, panel A2) was considerably diluted (37%) due to contribution from *de novo* biosynthesis. This further showed the presence of MIP synthase activity in the parasite.

Incorporation of D-[6-^{13}C]glucose in the parasite

Keeping in view the results obtained from *myo*-[1-^{13}C]inositol incorporation and to further complement the existence of a *de novo* biosynthetic route for inositol, the *Leishmania* promastigotes were starved in glucose-free Dulbecco's modified Eagle medium (DMEM) and then incubated with D-[6-^{13}C]-glucose (99 atom-%) along with a parallel control experiment. ESMS data of glycero-PI from labelling experiment showed that the ^{13}C label from precursor D-[6-^{13}C]glucose was incorporated into glycero-PI and the relative abundance of peak at *m/z* 334 was found to be 28% more in glycero-PI from the labelling experiment (Fig. 2, panel D2) than the glycero-PI from the control experiment (Fig. 2, panel D1). ESMS of the inositol samples obtained by complete hydrolysis of glycero-PI (from control as well as labelling experiments) showed that the relative abundance of peak *m/z* 180 was 27% more in inositol obtained by hydrolysis of glycero-PI from the labelling experiment (Fig. 1, panel E2) than the inositol from the control experiment (Fig. 1, panel E1). These glucose labellings unambiguously confirmed the results obtained from inositol-labelling experiments.

In summary, we have synthesised 1D-*myo*-[1-^{13}C]inositol by two independent routes and incorporated this into *Leishmania donovani* parasites. The isotopomeric analysis by negative-ion ESMS showed substantial dilution of ^{13}C atom-% in the biosynthetic glycero-PI and inositol products showing the presence of a *de novo* inositol biosynthesis in the parasite. This result was corroborated by a separate biosynthetic experiment using D-[6-^{13}C]glucose as precursor. This led to the identification of MIP synthase activity in the parasite which could be exploited for drug design. We have also demonstrated the potential of the combined application of stable isotope labelling and ESMS for the elucidation of biosynthetic pathways in parasites where this approach has not been exploited.

Experimental

General methods

The chemicals and reagents used for synthesis of labelled materials were from Aldrich and Fluka, D-[6-^{13}C]glucose (99 atom-% enriched) was from Aldrich. The precoated TLC and HPTLC plates were from Merck. Culture media (Medium-199, DMEM) and other additives/vitamins/co-factors used in *Leishmania* parasite culture were from Gibco-BRL. Radiolabelled metabolic precursors, [U-^{14}C]glycerol (147.8 mCi mmol^{-1}), D-[1-^{14}C]glucosamine (52.6 mCi mmol^{-1}), *N*-acetyl-D-[1-^{3}H]glucosamine (3.0 Ci mmol^{-1}), [1-^{14}C]palmitic acid (54 mCi mmol^{-1}), and En^{3}Hance spray were from DuPont-NEN,

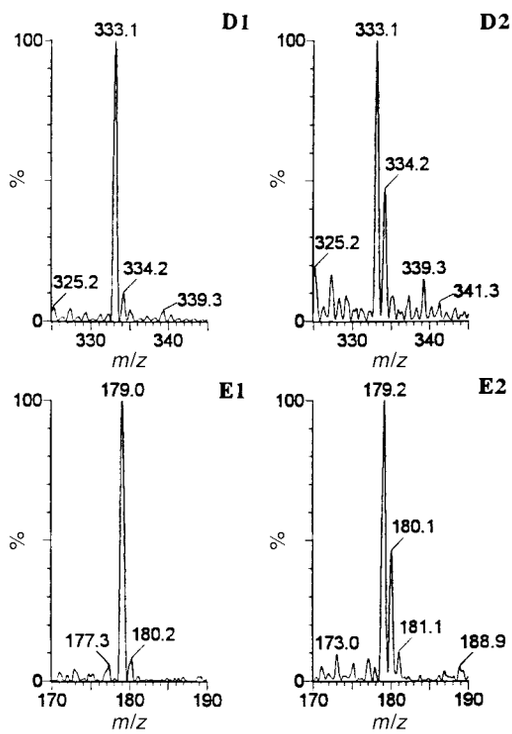


Fig. 2 ESMS (negative-ion) data from experiment on biosynthetic incorporation of D-[6-^{13}C]glucose in *Leishmania donovani*: (**D1**) ESMS of glycero-PI purified from control experiment; (**D2**) glycero-PI purified from labelling experiment; (**E1**) ESMS of inositol obtained by hydrolysis of glycero-PI from control experiment; (**E2**) inositol obtained by hydrolysis of glycero-PI from labelling experiment.

and D-[2-^{3}H]mannose (15 Ci mmol^{-1}) and *myo*-[^{3}H]inositol (83 Ci mmol^{-1}) from Amersham. The enzymes phospholipase A_2 (bee venom), PI-specific phospholipase C (*Bacillus cereus*) and other biochemicals were from Sigma. The negative-ion ESMS data was obtained on a VG Platform-II quadrupole mass spectrometer equipped with a MassLynx™ data system and pneumatic-nebuliser-assisted electrospray LC/MS interface. Acetonitrile–water (1:1 mixture) was used as carrier solvent at a flow rate of 10 μl min^{-1} and the analyte was infused through a rheodyne injector into the ESMS probe. NMR spectra were recorded on a Bruker DRX spectrometer operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C and 125 MHz for ^{31}P nuclei. Unless stated otherwise, the spectra were recorded in $CDCl_3$ and chemical shifts are in ppm relative to residual solvent resonance. *J*-Values are given in Hz. The NMR spectral assignments were made using COSY, HETCOR and HMQC experiments.

Enzymic synthesis of *myo*-inositol

Glucose 6-phosphate 2. D-Glucose **1** (77.4 mg, 0.43 mmol) was dissolved in deoxygenated water (2 ml) and $MgCl_2 \cdot 6H_2O$ (5.1 mg, 0.25 mmol), KCl (3.73 mg, 0.05 mmol), dithiothreitol (DTT) (2.3 mg, 0.015 mmol) and sodium azide (0.65 mg, 0.01 mmol) were added. The mixture was stirred for 10 min at room temperature and then ATP (272.3 mg, 0.45 mmol; USB) was added, the pH was adjusted to 7.6 with 1 M KOH and the

solution was purged with nitrogen for 5 min. Now, hexokinase (Type VI from baker's yeast, 40 U; Sigma) solution in water (200 μl) was added and the mixture was incubated at 37 °C. The progress of the phosphorylation was checked by TLC in 9.5 mM tetrabutylammonium hydroxide in 80% aq. acetonitrile and spots were visualised by ammonium molybdate–cerium(IV) sulfate reagent. The reaction mixture turned turbid at the end of the reaction, and the mixture was brought to pH 9.0 with 1 M KOH, and barium acetate (200 mg in 600 μl of water) was added with stirring. The precipitate was removed by centrifugation (3500 rpm for 5 min at 20 °C) and the supernatant was mixed with cation-exchange resin (100 mg, Dowex 50 W, HCR W2 activated with 1 M HCl, and washed with water), and the resultant clear solution was decanted and its pH adjusted to 9.0. MeOH was then added dropwise to the brink of precipitation. This solution was applied to an anion-exchange resin column (Dowex 1X8, 25–50 mesh; 1 × 10 cm bed, washed with water, 50% aq. methanol, 65 mM NH_4HCO_3 and again with water). Elution was performed with water (50 ml), 50% aq. methanol (50 ml), 65 mM NH_4HCO_3 in 50% aq. methanol and finally with 200 mM aq. NH_4HCO_3. This last fraction was lyophilised to constant weight to give D-glucose 6-phosphate as its diammonium salt (103 mg, 81%) as a white powder. This compound was analyzed by TLC [9.5 mM tetrabutylammonium hydroxide in 80% aq. acetonitrile; spots visualised by ammonium molybdate–cerium(IV) sulfate reagent, R_f 0.33]; ^{1}H NMR (D_2O) δ 5.07 (1H, d, J 3.6, H-1α), 4.49 (1H, d, J 8.0, H-1β), 4.08–3.92 (2H, m, H_2-6), 3.59–3.33 (3H, m); ^{31}P NMR δ 4.20, assignments confirmed by COSY; ESMS$^-$ m/z 259.2 $[M - H]^-$.

Culture of mutant (*opi 1δ*) strain of yeast, and partial purification of MIP synthase. The mutant yeast strain (*Saccharomyces cerevisiae*) was obtained from American Type Culture Collection (ATCC) maintained on agar slants. This is an overproducer of inositol with the genotype *MATa, opi1* (also termed *opi 1δ*). The prepared agar slants were streaked with the yeast and maintained at 4 °C. For YEPD medium preparation, yeast extract (1 g), peptone (2 g), and agar (1.3 g) were dissolved in 90 ml of water, and the solution was boiled for 5 min, allowed to cool to about 70 °C, and glucose (2 g in 10 ml of water) was added. The medium was dispensed at 6 ml tube^{-1} in autoclaved tubes which were laid in a slanting position and allowed to solidify at ambient temperature. For bulk culture of the yeast, an inositol-deficient medium containing yeast extract–peptone–dextrose (YEPD medium) was prepared, because in inositol-rich medium the turnover of MIP synthase was low. For this reason, yeast extract (1.2 g, 0.1%) and peptone (24 g, 2%) were dissolved in water (1100 ml; pH 6.0) in a conical flask fitted with an air-bubbling assembly. The assembly and medium was autoclaved at 121 °C at 15 psi for 15 min and the sterilised solution was cooled to ≈75 °C and glucose solution (24 g in 100 ml of filter-sterilised water) was added. The medium was inoculated with mutant yeast and the culture was incubated at 30 °C for 48 h with bubbling of air. After 48 h of incubation, the cells were harvested (centrifugation at 4 °C and 4000 rpm for 30 min) and washed twice with 100 ml of buffer A [Tris·HCl (20 mM, pH 7.2; Sigma) containing ammonium chloride (20 mM), 2-mercaptoethanol (10 mM) and phenylmethanelsulfonyl fluoride (PMSF, 0.5 mM; Sigma)], each time pelleting the cells at 6000 rpm for 10 min at 4 °C. The final washed pellet was resuspended in 40 ml of buffer A. An aliquot of this suspension was diluted 20-fold and used for microscopic examination for intactness of cells and any possible presence of microbial contaminants. The suspension was subjected to disruption by a French Press (SLM-AMINCO Spectronic Instruments). The disruption chamber was pre-cooled to below 0 °C into which the suspension (20 ml) was added, a pressure of 40 000 psi was applied, and the cycle was repeated thrice with each 20 ml part. The final disrupted material was pooled and checked under a microscope for the extent of disruption. The cell debris was removed from the lysate by centrifugation at 13 000 *g* (Kontron) for 20 min and the clear supernatant was taken. The nucleic acids were precipitated by slow addition of 2.22 ml of 25% (w/v) streptomycin sulfate solution to this supernatant at 0 °C and storage for 30 min. The precipitate was removed by centrifugation at 27 000 *g* for 20 min. To the clear supernatant (30 ml) was added powdered ammonium sulfate (6.87 g) slowly with stirring at 0 °C to a final concentration of 229 g l^{-1} (40% saturation). After being stirred for another 30 min the solution was left at 4 °C for 2 h and precipitated material was removed by centrifugation (27 000 *g*; 20 min). Salt saturation was increased to 75% (483 g l^{-1}) by adding ammonium sulfate (6.68 g), and after stirring for another 30 min the solution was left at 4 °C for 2 h and the precipitated material was obtained by centrifugation (27 000 *g*, 20 min). This final pellet (600 mg) was resuspended in 15 ml of buffer A and dialysed against buffer A (1 1 × 4) at 4 °C using 12 000 MW dialysis membrane (Sigma). Final dialysed material was checked for enzyme activity, protein concentration (BCA method) and SDS-PAGE and stored at −20 °C.

***myo*-Inositol 1-phosphate 3.** D-Glucose 6-phosphate diammonium salt **2** (90 mg, 0.306 mmol) was dissolved in 10 ml of buffer B (50 mM Tris·HCl, pH 7.4, containing 5% ethanol, 0.02% sodium azide, 14.4 mM NH_4Cl, 22 mM NaF and 10 mM DTT). Nicotinamide adenine dinucleotide (β-NAD^+, 114.7 mg, 0.173 mmol; Sigma) and partially purified MIP synthase (2.5 ml solution) was added and the mixture was incubated at 37 °C for 24 h. The progress of the reaction was monitored by TLC (9.5 mM tetrabutylammonium hydroxide in 80% aq. acetonitrile). At the end of the reaction, the mixture was heated at 95 °C for 3 min and the denatured precipitated protein was removed by centrifugation for 5 min at 5000 *g*. The supernatant was applied to an anion-exchange resin column [AG1-X8; Bio-Rad, bed 1 × 15 cm, resin washed with water and equilibrated with buffer C (100 mM ammonium formate containing 20 mM sodium tetraborate, pH 8.5)]. Elution was performed with a linear gradient (100 ml) of buffer C to buffer D (625 mM ammonium formate containing 20 mM sodium tetraborate, pH 8.5). Fractions (5 ml) were collected, and checked by TLC, and the fractions containing inositol 1-phosphate were pooled. From this desired fraction, cations were removed by treatment with a cation-exchange resin (Dowex HCR W2; Sigma, bed 1 × 10 cm, resin washed with 2 M HCl and water). Elution was performed with water and a single fraction was obtained from effluent pH 1.0 to 6.0. This fraction was lyophilised and the residue was repeatedly dissolved in methanol and lyophilised to constant weight. The resulting product **3** (17.2 mg, 21.7%) showed ^{1}H NMR (D_2O) δ 4.23 (1H, t, J 2.7, H-2), 3.90 (1H, td, J 9.7 and 2.7, H-1), 3.74 (1H, t, J 9.5, H-6), 3.64 (1H, t, J 9.8, H-4), 3.58 (1H, dd, J 9.8 and 2.7, H-3), 3.34 (1H, t, J 7, H-5) and assignments were confirmed by a COSY experiment; ^{31}P NMR δ 2.73; ESMS$^-$ m/z 259.2 $[M - H]^-$.

Dephosphorylation of *myo*-inositol 1-phosphate. Compound **3** (17.2 mg, 66 mmol) was dissolved in buffer (3 ml; 0.1 M Tris·HCl, pH 8.0) and alkaline phosphatase (4 U; 8.4 ml, stock 82 U 170 ml^{-1}, Type III from *E. coli*; Sigma) was added. The reaction mixture was incubated at 25 °C for 16 h after which the entire reaction mixture was loaded onto a silica column (1 × 25 cm, packed in 20% methanol in chloroform). Elution was as follows: fractions 1–4 (7.5 ml each) with 20% methanol in chloroform, fractions 5–12 (7.5 ml each) with 30% methanol in chloroform, fractions 13–19 (7.5 ml each) with 50% methanol in chloroform, fractions 20–21 (10 ml each) with methanol, and fraction 22 (10 ml) with water. The fractions were analyzed by TLC (9.5 mM tetrabutylammonium hydroxide in 80% aq. acetonitrile; spots visualised by potassium permanganate reagent, R_f 0.15). Only water fractions contained

myo-inositol **4**, which was lyophilised (6.7 mg, pure, 63.6%); ^{1}H NMR (D_2O) δ 3.96 (1H, t, *J* 2.7, H-2), 3.53 (2H, t, *J* 9.8, H-4, -6), 3.44 (2H, m, H-1, -3), 3.18 (1H, t, *J* 9.2, H-5), with assignments confirmed by a COSY experiment; ESMS$^-$ *m*/*z* 179 $[M - H]^-$.

Chemical synthesis of D-*myo*-inositol

Methyl 2,3,4-tri-*O*-benzyl-6-*O*-(triphenylmethyl)-D-glucopyranoside 5. A solution of D-glucose **1** (200 mg. 1.11 mmol) in anhydrous MeOH (5 ml) was mixed with cation-exchange resin (Dowex 50W; 200 mg, resin pre-washed with water, 2 M HCl, water and MeOH) and the mixture was refluxed overnight. It was cooled to room temperature and the resin was removed by filtration, and the filtrate was concentrated to give viscous, solid methyl α-D-glucopyranoside (208 mg, 96%). Methyl glucoside (194 mg, 1 mmol) was dissolved in DMF (0.6 ml), trityl chloride (307 mg, 1.1 mmol), DMAP (20 mg, 0.08 mmol) and triethylamine (153 µl, 1.1 mmol) were added, and the mixture was stirred overnight at room temp. After completion of the reaction the mixture was concentrated, and purified by silica column chromatography to give methyl 6-*O*-(triphenylmethyl)-D-glucopyranoside (270 mg, 62%). This intermediate (218 mg, 0.5 mmol) and NaH (144 mg, 3.6 mmol) were dissolved in DMF (1.5 ml), and benzyl bromide (522 mg, 363 µl, 3.05 mmol) was added dropwise at 0 °C. The reaction mixture was stirred overnight at room temp. and excess of NaH was destroyed by addition of MeOH and water, and the mixture extracted with $CHCl_3$. The organic phase was washed with water and dried (Na_2SO_4), concentrated, and purified by silica column chromatography to give **5** [17] (251 mg, 71%); ^{1}H NMR δ 7.55–6.8 (30H, m, Ph), 5.1–4.2 (7H, m, 3 × PhCH_2 and H-1), 4.15–3.35 (5H, m), 3.40 (3H, s), 3.20 (1H, dd, *J* 10 and 4, H-2); ESMS$^+$ *m*/*z* 729 $[M + Na]^+$.

Methyl 2,3,4-tri-*O*-benzyl-D-glucopyranoside 6. To a solution of compound **5** (212 mg, 0.3 mmol) in dichloromethane–methanol (1 : 2; 3 ml) was added *p*-TsOH (3 mg) and the mixture was stirred overnight. Usual work-up and purification on a silica column gave a colourless, syrupy material **6** [14] (128 mg, 92%); ^{1}H NMR δ 7.5–7.1 (15H, m, Ph), 5.1–4.5 (6H, m, 3 × PhCH_2), 4.57 (1H, d, *J* 2, H-1), 4.02 (1H, dd, *J* 9 and 7.5, H-3), 3.84–3.37 (5H, m), 3.37 (3H, s, OMe); ^{13}C NMR δ 183.03, 128.38–127.52 (7 peaks clustered together), 98.09, 81.85, 79.91, 77.34, 75.64, 74.92, 73.32, 70.58, 61.78, 55.09; ESMS$^+$ *m*/*z* 487 $[M + Na]^+$.

Methyl 2,3,4-tri-*O*-benzyl-6-*O*-acetyl-D-*xylo*-pent-5-enopyranoside 8. A solution of oxalyl dichloride (10.16 mg, 7 µl, 0.08 mmol) in anhydrous CH_2Cl_2 (2.78 ml) was cooled to −78 °C and DMSO (12.5 mg, 11.4 µl, 0.16 mmol) was added dropwise, followed by addition of a solution of **6** (93 mg, 0.2 mmol) in CH_2Cl_2 (926 µl) over a period of 5 min. The mixture was stirred for 30 min and then triethylamine (222 µl) was added. The solution was brought to room temp., water (2 ml) was added, and the mixture was extracted with CH_2Cl_2. The organic layer was dried over sodium sulfate to give intermediate aldehyde **7** which was used directly and dissolved in dry acetonitrile (2.5 ml) and anhydrous K_2CO_3 (171 mg, 1.24 mmol) was added. After stirring of the mixture for 10 min at room temp., Ac_2O (111 µl, 1.17 mmol) was added and the reaction mixture was heated overnight under nitrogen at 80 °C. After completion, water (2.5 ml) was added and the mixture was extracted with diethyl ether. The organic layer was concentrated and subjected to silica column chromatography and elute with 20–30% ethyl acetate–hexane to provide the desired product methyl 2,3,4-tri-*O*-benzyl-6-*O*-acetyl-D-*xylo*-pent-5-enopyranoside [14] **8** (54 mg, 53%); ^{1}H NMR δ 7.4–7.2 (15H, m, Ph), 4.9–4.6 (8H, m), 4.0–3.9 (2H, m), 3.6–3.5 (1H, m), 3.47 (3H, s), 2.15 (3H, s); ^{13}C NMR δ 167.21, 134.92, 128.46–127.56 (peaks clustered together), 123.02, 99.70, 81.20, 79.02, 77.71, 75.58, 74.40, 73.62, 73.39, 73.16, 72.51, 56.14, 20.49; ESMS$^+$ *m*/*z* 527 $[M + Na]^+$.

1-*O*-Acetyl-3,4,5-tri-*O*-benzyl-6-deoxy-6-oxo-*myo*-inositol 9. Enol acetate **8** (50.4 mg, 0.1 mmol) was dissolved in acetone (2 ml)–water (800 µl), and mercury(II) acetate (310 mg, 0.99 mmol) was added. The solution was stirred for 45 min and then saturated aq. NaCl (1.2 ml) was added, and the mixture was stirred for 24 h at room temp. The solvents were evaporated off and the residue was extracted with ethyl acetate. The organic layer was dried, concentrated and purified by silica column chromatography to provide desired **9** [14] (21 mg, 42%); ^{1}H NMR δ 7.4–7.2 (15H, m, Ph), 5.17 (1H, br s, H-1), 4.95–4.7 (5H, m), 4.52 (1H, d, *J* 11), 4.34 (1H, t, *J* 2.6), 4.17–4.05 (2H, m), 3.88–3.84 (1H, dd, *J* 6 and 2.4), 2.24 (3H, s); ^{13}C NMR δ 197.90, 169.85, 128.53–127.67 (peaks clustered together), 83.44, 81.72, 78.87, 76.05, 74.94, 73.48, 73.21, 69.21, 20.43; ESMS$^+$ *m*/*z* 513 $[M + Na]^+$.

1-*O*-Acetyl-3,4,5-tri-*O*-benzyl-*myo*-inositol 10. To a solution of 1-*O*-acetyl-3,4,5-tri-*O*-benzyl-6-deoxy-6-oxo-*myo*-inositol **9** (19.6 mg, 0.04 mmol) in anhydrous acetonitrile (1.2 ml) was added sodium triacetoxyborohydride (86.4 mg, 0.4 mmol, freshly prepared) and 200 µl of glacial acetic acid. The mixture was stirred at room temp. for 45 min and then 0.5 M sodium hydrogen sulfate (720 µl) was added dropwise to destroy excess of hydride reagent. The mixture was extracted with ethyl acetate and the organic phase was washed successively with 0.5 M sodium hydrogen sulfate and saturated aq. disodium hydrogen phosphate, dried over sodium sulfate, and concentrated to give **10** [14] (13.5 mg, 68.6%); ^{1}H NMR δ 7.4–7.2 (15H, m, Ph), 5.18 (1H, dd, $J_{1,2}$ 3, $J_{1,6}$ 9, H-1), 4.90–4.50 (6H, m, 3 × PhCH_2), 4.38 (1H, t, *J* 3, H-2), 4.15 (1H, t, $J_{6,5}$ 10, $J_{6,1}$ 9, H-6), 3.97 (1H, t, $J_{4,5}$ 9, $J_{4,3}$ 10, H-4), 3.59 (1H, dd, $J_{3,4}$ 10, $J_{3,2}$ 3, H-3), 4.26 (1H, dd, $J_{5,4}$ 9, $J_{5,6}$ 10, H-5), 2.24 (3H, s, COMe); ^{13}C NMR δ 170.82, 128.50–127.59 (peaks clustered together), 82.90, 80.83, 80.05, 75.72, 75.55, 73.10, 72.72, 70.32, 67.67, 22.62; chemical shifts and stereochemical assignments were confirmed by COSY and NOESY experiments; ESMS$^+$ *m*/*z* 515 $[M + Na]^+$.

1D-1-*O*-Acetyl-*myo*-inositol 11. 1-*O*-Acetyl-3,4,5-tri-*O*-benzyl-*myo*-inositol **10** (12.3 mg, 0.025 mmol) and 10% Pd/C (10 mg) as a mixture in ethanol (1.5 ml) was hydrogenated (10 atm) in a Parr cavitation apparatus for 24 h. After completion of the reaction, the catalyst was filtered off through a Celite pad and washed with ethanol–water (2 : 1) and the pH of the filtrate was brought to 8 by NH_4OH. The solution was concentrated to afford 1-*O*-acetyl-*myo*-inositol **11** (5.1 mg, 92%); ^{1}H NMR (D_2O) δ 4.65 (1H, br s, H-1), 4.03 (1H, t, *J* 2.7, H-2), 3.9–3.2 (4H, m, H-3, -4, -5 and -6), 2.03 (3H, s); ESMS$^-$ *m*/*z* 221.3 $[M - H]^-$.

D-*myo*-Inositol 4. 1-*O*-Acetyl-*myo*-inositol (5 mg, 22.5 µmol) was dissolved in dry methanol (2 ml) and sodium methoxide (2.4 mg, 45 µmol) was added. The mixture was heated at 70 °C for 3 h after which the excess of NaOMe was destroyed by addition of water (200 µl). The contents were evaporated to dryness and then reconstituted in water (1 ml), cation-exchange resin (Amberlite IR-120, pre-treated with 2 M HCl, and water) was added, and the mixture was vortexed. The resin was removed by filtration and the filtrate was lyophilised to yield white, powdery D-*myo*-inositol (3.8 mg, 94%), R_f 0.15 in 9.5 mM tetrabutylammonium hydroxide in 80% aq. acetonitrile; spot visualised by ammonium molybdate–cerium(IV) sulfate; ^{1}H NMR (D_2O) δ 3.96 (1H, t, *J* 2.7, H-2), 3.53 (2H, t, *J* 9.2 and 9.8, H-4, -6), 3.44 (2H, dd, *J* 9.8 and 2.7, H-1, -3), 3.18 (1H, dd, *J* 9.2 and 9.8, H-5); ^{13}C NMR δ 74.14 (C-5), 72.19 (C-4, -6), 71.97 (C-2), 70.92 (C-1, -3); assignments were con-

firmed by COSY and HMQC experiments; ESMS$^-$ *m/z* 179 $[M - H]^-$.

Synthesis of ^{13}C-labelled *myo*-inositol 4

myo-[1-^{13}C]Inositol was synthesised from D-[6-^{13}C]glucose as starting material (200 mg, 99 atom-%, Aldrich) which was taken through all the steps described above for the unlabelled enzymic as well as chemical syntheses. The progress of reactions was monitored by comparing TLC results with the corresponding unlabelled compounds. From the chemical route 22.7 mg of labelled inositol could be obtained from 200 mg of D-[6-^{13}C]glucose. The final labelled *myo*-[1-^{13}C]inositol was characterised by ^{1}H NMR (D_2O) δ 4.0 (1H, m, H-2, $^3J_{H\text{-}H}$ 2.7, $^2J_{C\text{-}H}$ 5.2 due to coupling of H-2 with ^{13}C-enriched C-1), 3.50 (2H, m, H-4 and -6, *J* 9.8, 9.2 and $^2J_{C\text{-}H}$ 5.2 due to coupling of H-6 with enriched ^{13}C-1), 3.40 (2H, m, H-1, -3, $J_{C\text{-}H}$ 141.5 Hz due to direct coupling of enriched ^{13}C-1 and H-1), 3.10 (1H, t, *J* 9.2, H-5); ^{13}C NMR (proton-decoupled) δ 71.0 (^{13}C-enriched C-1); ^{13}C NMR (proton-coupled) δ 71.0 (d, *J* 141.5 due to direct ^{13}C-1/H-1 coupling; each peak of this doublet was further split by 5.2 Hz due to $^2J_{C\text{-}H}$ coupling of ^{13}C-1 with H-2 and -6); the ^{1}H and ^{13}C assignments were confirmed by COSY, HETCOR and HMQC experiments; ESMS$^-$ *m/z* 180 $[M - H]^-$.

Microbiological methods

Parasite culture. *Leishmania donovani* promastigotes (DD8 strain, obtained from Professor K.-P. Chang of Chicago Medical School) were grown at 23 °C in Medium-199 supplemented with 10% HI-FBS (heat-inactivated foetal bovine serum), Hepes (25 mM) and gentamycin sulfate (50 mg l^{-1}) to a density of 4×10^7 cells ml^{-1}. The biosynthetic viability of the cell culture was evaluated by metabolic labelling of *L. donovani* promastigotes with 2.5 μCi of D-[1-^{14}C]glucosamine, 25 μCi of *N*-acetyl-D-[1-^{3}H]glucosamine, 2.5 μCi of [1-^{14}C]palmitic acid, 21.2 μCi of D-[2-^{3}H]mannose and 15 μCi of *myo*-[^{3}H]inositol. In the case of labelling with mannose, glucosamine and *N*-acetylglucosamine, the precursors were added to three tubes separately, each containing 10^9 promastigotes (no starvation) in 2 ml of DMEM. The modified medium (dDME) supplemented with 0.3% bovine serum albumin (fraction V), adenosine (0.05 mM), xanthine (0.05 mM), biotin (10 mg l^{-1}), Tween 80 (40 mg l^{-1}), haemin (5 mg l^{-1}), and triethanolamine (0.5 ml l^{-1}) was prepared according to the method of Orlandi and Turco.[20] In the case of inositol labelling, promastigotes were starved in dDME (without inositol and with fat-free BSA; prepared from inositol-free DMEM) for 48 h at 23 °C and then to this washed, resuspended cell pellet (4.75×10^{10} promastigotes in 5 ml of dDME) was added *myo*-[^{3}H]inositol. Similarly, for labelling with palmitic acid, cells were starved in dDME (with fat-free BSA; prepared from pyruvate-free DMEM) for 48 h at 23 °C and then to the washed, resuspended cell pellet (10^9 promastigotes in 5 ml of the same dDME) was added [1-^{14}C]palmitic acid. Suitable controls were used in each of the above metabolic labelling experiments. Incubations were carried out overnight at 23 °C and cell viability was checked before and after incubation.

For biosynthetic incorporation, exponentially growing parasite cells from Medium-199 were harvested by centrifuging at 4000 rpm for 8 min at 23 °C. The cells were starved in 20 ml of inositol-free dDME with fat-free BSA at 23 °C for 24 h after which the culture was harvested. The cell suspensions were checked for parasite viability before and after incubation. After completion of incubation, the parasites were collected and PI/GPIs isolated. The cell pellet was washed three times with phosphate-buffered saline (PBS) (50 mM, pH 7.2). The washed cell pellet was extracted twice with 10 volumes of chloroform–methanol–water (1 : 2 : 0.8, v/v) and the insoluble material was removed by centrifugation (10 000 *g*; 15 min). The combined supernatants were dried under a stream of nitrogen and the extracted lipids were partitioned between water and butan-1-ol (1 : 2, v/v). The butanol phase was removed and the lower aqueous phase was re-extracted with water-saturated butan-1-ol. The pooled butanol phase containing PI/GPIs was concentrated and analysed by HPTLC using a $CHCl_3$–MeOH–13 M NH_3–1 M NH_4OAc–H_2O (180 : 140 : 9 : 9 : 23) solvent system. The presence of PI and GPI bands was confirmed by exposing the plates to Bial's reagent (0.9% $FeCl_3$ and 0.55% orcinol in ethanol–H_2SO_4) for the presence of sugars, DPH reagent (0.03% 1,6-diphenylhexa-2,3,5-triene in $CHCl_3$) for lipids, and Molybdenum Blue spray reagent (zinzade reagent) for phospholipids. The identities of the isolated parasitic PI and GPI molecules were confirmed by comparison with authentic samples. To locate positions of radio-labelled glycolipids, the HPTLC plate was sprayed with En^{3}Hance (DuPont NEN) and then used for fluorography on Kodak film (exposed at −70 °C). Isolated compounds were subjected to PI-specific phospholipase C (PI-PLC, *Bacillus cereus*), phospholipase A_2 enzyme treatment, nitrous acid deamination, negative-ion ESMS, and NMR for confirmation of the structure.

Preparation of glycero-PI by mild alkali hydrolysis. Parasitic PI fraction (50 μl) was dried with a stream of nitrogen gas and 4 M NH_4OH (100 μl) was added. The mixture was incubated at 37 °C for 16 h. At the end of reaction butan-1-ol (100 μl) was added, the contents were thoroughly mixed, and the phases were separated by centrifugation (10 000 rpm for 5 min at 20 °C). The aqueous layer was re-extracted with water-saturated butan-1-ol (100 μl). The final aqueous phase was freeze-dried to obtain glycero-PI which was used for ESMS (continuum data, *m/z* 200–500).

Similarly, mild alkaline hydrolysis of PI (Sigma) (PC, egg yolk), lecithin (soybean, USB) and a 1 : 1 mixture of pure PI and PC was carried out with 4 M NH_4OH, and at the end of the reaction $CHCl_3$ (100 μl) was added to each tube and the contents mixed thoroughly. The organic and aqueous phases were separated by centrifugation, concentrated, and used for ESMS.

Hydrolysis of glycero-PI to inositol. The glycero-PI from the 4 M NH_4OH digest of the parasite PI was lyophilised in a glass tube, 10% NH_4OH (100 μl) was added, and the tube was sealed under nitrogen before being heated at 150 °C for 18 h; after cooling, the tube was broken and the contents were mixed with $CHCl_3$ (100 μl). The aqueous layer was freeze-dried and further purified for isolation of labelled inositol by PLC (9.5 mM tetrabutylammonium hydroxide in 80% acetonitrile–water; R_f inositol 0.15, and glucose 0.34).

Biosynthetic incorporation of 1D-*myo*-[1-^{13}C]inositol in *L. donovani*

Exponentially growing *L. donovani* promastigotes were harvested from Medium-199, washed thrice, and resuspended in inositol-free Medium-199 with fat-free BSA. The promastigotes were starved for 24 h at 23 °C after which the culture was divided into two equal parts and each was harvested separately to give 2×10^9 cells $part^{-1}$. To the first part was added 5 ml of normal medium with fat-free BSA (*control experiment*) and to the second part were added 5 ml of *myo*-[1-^{13}C]inositol-rich medium with fat-free BSA and 2 mg of labelled inositol (50 atom-% ^{13}C) (*labelling experiment*). The cells were incubated at 23 °C for 24 h, after which harvesting, initial processing, and extraction of PI were carried out. The PI fraction in each case was divided into two equal parts, one of which was treated with 4 M NH_4OH at room temp., and the other part was treated with 10% NH_4OH at 150 °C in a sealed tube. After both treatments the aqueous phases were separated, washed with $CHCl_3$–MeOH, and purified for glycero-PI and inositol, respectively.

At zero time of incubation, a 50 μl aliquot of the culture was removed and processed as described above for the isolation of free inositol, which was further subjected to PLC (9.5 mM

tetrabutylammonium hydroxide in H_2O–CH_3CN 20:80). This was required to separate inositol (R_f 0.15) from glucose (R_f 0.34) present in the parasite culture. The inositol band was scraped from the plates and eluted in 20% methanol in water by sonication. The silica particles were removed by centrifugation at 13 000 rpm for 20 min and the supernatant was used for ESMS.

Biosynthetic incorporation of D-[1^{13}C]glucose in *L. donovani*

Exponentially growing *L. donovani* promastigotes were harvested from Medium-199 and washed thrice with glucose-free DMEM. The washed pellet was resuspended in 50 ml of glucose-free dDME. The cells were starved for 24 h at 23 °C after which the suspension was divided into two equal parts and each part was harvested separately to give 6×10^8 cells part^{-1}. To each part was added 5 ml of glucose-free dDME. Further, the first part (*control*) was fortified with 22.5 mg of D-glucose while the other part (*labelling*) was fortified with 22.5 mg of D-[6-^{13}C]glucose (99 atom-%). These cell suspensions were incubated for 24 h at 23 °C, and labelled glycero-PI and inositol were isolated as described above and analysed by ESMS.

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