Ppm1-Encoded Polyprenyl Monophosphomannose Synthase Activity Is Essential for Lipoglycan Synthesis and Survival in Mycobacteria

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Abstract

The biosynthesis of mycobacterial mannose-containing lipoglycans, such as lipomannan (LM) and the immunomodulator lipoarabinomannan (LAM), is carried out by the GT-C superfamily of glycosyltransferases that require polyprenylphosphate-based mannos (PPM) as a sugar donor. The essentiality of lipoglycan synthesis for growth makes the glycosyltransferase synthesizing PPM, a potential drug target in Mycobacterium tuberculosis, the causative agent of tuberculosis. In M. tuberculosis, PPM has been shown to be synthesized by Ppm1 in enzymatic assays. However, genetic evidence for its essentiality and in vivo role in LM/LAM and PPM biosynthesis is lacking. In this study, we demonstrate that MSMEG3859, a Mycobacterium smegmatis gene encoding the homologue of the catalytic domain of M. tuberculosis Ppm1, is essential for survival. Depletion of MSMEG3859 in a conditional mutant of M. smegmatis resulted in the loss of higher order phosphatidyl-myo-inositol mannosides (PIMs) and lipomannan. We were also able to demonstrate that two other M. tuberculosis genes encoding glycosyltransferases that either had been shown to possess PPM synthase activity (Rv3779), or were involved in synthesizing similar polyprenol-linked donors (ppgS), were unable to compensate for the loss of MSMEG3859 in the conditional mutant.

Introduction

Tuberculosis (TB) affects a third of mankind and causes 1.7 million fatalities annually [1]. The spread of TB has been facilitated in recent decades due to the susceptibility of HIV-infected individuals to Mycobacterium tuberculosis, the etiological agent of TB [2]. The problem has been compounded by the emergence of multi- and extensively-drug resistant M. tuberculosis strains [2]. Typically, the cell walls of the genus Mycobacterium contain mycolic acids (m), arabinogalactan (AG) and peptidoglycan (P), which are covalently linked to each other to form the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex [3]. A particular group of specialized glycolipopolisphidophospholipid, phosphatidyl-myo-inositol (PI) mannosides (PIMs) and lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), are found in the outer leaflet of mAGP [4]. LM and LAM, which are based on a core pilA gene encoding the homologue of the catalytic domain of M. tuberculosis Ppm1, are essential for survival. Depletion of MSMEG3859 in a conditional mutant of M. smegmatis resulted in the loss of higher order phosphatidyl-myo-inositol mannosides (PIMs) and lipomannan. We were also able to demonstrate that two other M. tuberculosis genes encoding glycosyltransferases that either had been shown to possess PPM synthase activity (Rv3779), or were involved in synthesizing similar polyprenol-linked donors (ppgS), were unable to compensate for the loss of MSMEG3859 in the conditional mutant.

ManLAM and PILAM exhibit a broad range of immunomodulatory activities. For example ManLAM, which is predominantly found in the slow-growing pathogenic mycobacteria, inhibits a number of immune system effector functions, including interferon-γ-mediated activation of macrophages [4]. ManLAM also inhibits the production of the pro-inflammatory cytokines interleukin-12 [4] and tumor necrosis factor-α [9]. PIMs, which are characteristic of the fast-growing saprophytic mycobacteria, can induce a pro-inflammatory response in a Toll-like 2 receptor-dependent manner [10].

The current model of lipoglycan biosynthesis is supported by biochemical and genetic studies, and follows a linear pathway from PI→PIM1→LAM [4]. PimA (Rv2610c) and PimB (Rv2188), are α-mannopyranosyltransferases, belonging to the GT-B superfamily and utilize GDP-mannose [11,12], adding Manp residues at positions 0-2 and 0-6 of PI, respectively to produce PIM2, that can be subsequently acylated by Rv2611c, at the 6 position of the Manp residue to generate Ac1/2PIM2 [13]. RvD2-ORF1 from M. tuberculosis CDC1551, designated as PimC and an unknown glycosyltransferase (PimD), catalyzes the addition of a Manp residue from GDP-mannose to 6-OH of mannose at the non-reducing end of Ac1/2PIM2 to generate Ac1/2PIM3 [14] and Ac1/2PIM4, respectively. At this key junction point in the biosynthetic pathway, polyprenylphosphate-based mannos (PPM) donors are employed by the GT-C superfamily for elongation and
branching of Ac1/Ac2PIM4 leading to Ac1/Ac2PIM6, LM and LAM [15].

The pathway splits into two branches from Ac1/Ac2PIM4: one leads to the formation of Ac1/Ac2PIM6, whilst the other leads to the formation of LM and LAM. In the first branch, the protein consisting only of the C-terminal domain (Mt-ppm1/D2) of Mt-Ppm1/D2 has PPM synthase activity, and as a glycosyltransferase that uses polypropyl-P-D-GalNAc as a D-GalNp (or D-GalNAc) donor for transfer to 3,5-branched D-Araf residues of AG [30]. Additionally, the latter report also describes Rs6361 (ppgs) and Rs3632 as genes encoding a polypropyl-P-D-GalN synthase and a small integral membrane protein respectively, analogous to Ms-Ppm1/D2 and Ms-Ppm1/D1. While PpgS is a GT-2 family glycosyltransferase involved in the generation of polypropyl-phospho-N-acetylgalactosamine (polypropyl-P-GalNAc) from polypropyl-P and UDP-GalNAc, it could be envisaged as a secondary, potential PPM synthase.

In an effort to first confirm the in vivo role of Mt-Ppm1/D2 in LM/LAM biosynthesis we aimed to test the essentiality of PPM glycosyltransferase activity in M. smegmatis by using CESTET, a genetic tool for testing gene essentiality in M. smegmatis. As M. smegmatis does not encode homologues of ppgS or Rs3779, it also provided us with a valuable surrogate to probe the in vivo role and potential essentiality of MSMEG3859 in the absence of any potential functional redundancy caused by an alternative PPM synthase. Additionally, mutant or conditional mutant strains of M. smegmatis could subsequently be used as a host strain to test functional complementation of PPM synthase activity using recombinant ppgS or Rs3779.

Materials and Methods

Construction of Recombinant Plasmids

For generating an integrative vector containing MSMEG3859, the ORF was amplified from M. smegmatis mc²155 [31] genomic DNA using the primers F3859 (5'-TGGGAATTCCGCTGCGGTCAGCTGATGAGCGTCCCCG-3') and R3859 (5'-GCTATCGATTCAGCGGACCACGCCCCTG-3'), cloned downstream of the tetracycline promoter in the integrative vector pTIG6a vector (gift from A. Baughn and W.R. Jacobs Jr., Albert Einstein College of Medicine, NY) and named pTIG6a-MSMEG3859. For subsequent complementation/rescue experiments, the plasmid pMV261Apra, a derivative of pMV261 [32], was used for cloning various GTFs. MSMEG3859 was amplified using primer pairs F3859A (5'-GGCGGTCAGCTGCGGTCAGCTGATGAGCGTCCCCG-3') and R3859 (5'-GCTATCGATTCAGCGGACCACGCCCCTG-3'); the C-terminal domain of Rs2051c (Mt-ppm1/D2) was amplified using primer pairs F2051C (5'-TGGGAATTCCGCTGCGGTCAGCTGATGAGCGTCCCCG-3') and R2051C (5'-TGGGAATTCCGCTGCGGTCAGCTGATGAGCGTCCCCG-3') and R3779 using F3779 (5'-GATTGCGGTCAGCTGCGGTCAGCTGATGAGCGTCCCCG-3') and R3779 using F3779 (5'-GATTGCGGTCAGCTGCGGTCAGCTGATGAGCGTCCCCG-3'). The PCR amplified DNA fragments of MSMEG3859, Mt-ppm1/D2, R3779 and ppgS were cloned into pMV261Apra using primer-incorporated restriction sites and the resultant plasmids were named pAKR-MSMEG3859, pAKR-Mt-ppm1/D2, pAKR- MsPpm Conditional Mutant

The Ms. smegmatis conditional mutant MsPpm was generated using CESTET [33]. Briefly, a merodiploid was first generated by introducing pTIG6a-MSMEG3859 by electroporation into
M. smegmatis mc²155 [33]. The merodiploid strain mc²155::pTIC6a-MSMEG3859 was then subjected to specialized transduction as previously described [34] using a temperature-sensitive, recombinant phage phD_MsPpm designed to replace MSMEG3859-MSMEG3860 with a hygromycin resistance marker. Transductants were selected at the non-permissive temperature of 37°C on selective plates containing 25 mg/ml kanamycin, 100 mg/ml hygromycin B and 50 ng/ml anhydrotetracycline (ATc). After confirmation of gene replacement by Southern blot, one such transductant was named DMsPpm and was selected for further analysis.

Conditional Depletion of DMsPpm Conditional Mutant

The DMsPpm conditional mutant was grown in Tryptic Soy Broth (TSB; Difco) containing 0.05% Tween 80, 25 μg/ml kanamycin, 100 μg/ml hygromycin B and 50 ng/ml ATc and subsequently passaged twice in medium without ATc. To visualize the effects of the conditional depletion of MSMEG3859 in DMsPpm on lipids and lipoglycans, the strains were grown to OD 0.8, labelled with 10 μCi/ml glucose D-[¹⁴C(U)] (specific activity 250–360 mCi (9.25–13.3 GBq)/mmol; Perkin Elmer) and incubated at 37°C for 4 hours.

Extraction of Polar Lipids

Polar lipids and apolar lipids were extracted as described previously [35]. Briefly, cells from a 10 ml volume culture were washed once with 2 ml phosphate buffer saline (PBS) and treated with 2 ml CH₃OH:0.3% NaCl(aq) (100:10, v/v) and 2 ml petroleum ether for 30 min. The suspension was centrifuged and the upper layer containing apolar lipids was separated. An additional 2 ml of petroleum ether was added, mixed and centrifuged as described above and the two upper apolar lipid fractions were combined and dried. For polar lipids, 2.3 ml of CHCl₃:CH₃OH:0.3% NaCl(aq) (90:100:30, v/v/v) was added to the cell pellet and mixed for 1 h. This mixture was centrifuged and the supernatant was separated. The remaining cell pellet was mixed with 750 μl of CHCl₃:CH₃OH:0.3% NaCl(aq) (50:100:40, v/v/v) for 30 min, centrifuged and the supernatant was combined to the previous fraction. After repeating this step, 1.3 ml of CHCl₃ and 1.3 ml of 0.3% NaCl(aq) was mixed with the pooled supernatant, centrifuged, and the lower layer containing the polar lipids was recovered and dried. The polar lipid extracts were dried and resuspended in CHCl₃:CH₃OH (2:1, v/v), and incorporation of glucose D-[¹⁴C(U)] was quantified by liquid scintillation counting using 5% of the lipid fractions in 5 ml EcoScint A (National Diagnostics). Equal counts of polar lipid extracts (50 000 cpm) were applied to Silica Gel 60 F₂₅₄ (Merck 5554).

Figure 1. Genomic organization of ppm1 region in different mycobacteria and in Corynebacterium glutamicum. Homologous genes are indicated by similar arrows and pseudogenes in M. leprae are indicated by arrows with dotted borders. In M. tuberculosis and M..bovis, the ppm1-encoded protein consist of two domains fused together, while these two domains are encoded by two distinct ORFs in M. leprae, M. smegmatis and C. glutamicum.

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aluminium-backed TLC plates and developed using solvent system E for polar lipids: CHCl₃:CH₃OH:H₂O (60:30:6, v/v/v) in the first direction and CHCl₃:CH₃CO₂H:H₂O:CH₃OH (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film).

Extraction and Purification of Lipoglycans

Lipoglycans were extracted as described previously [36]. Briefly, dried cells from a 10 ml volume culture were resuspended in water and refulx five times with equal volume of 50% C₂H₅OH at 85 °C, for 6 h intervals, followed by centrifugation and recovery of the supernatant. The combined supernatants were dried and subjected to hot phenol-H₂O treatment at 65 °C. The aqueous phase containing the crude lipoglycan fraction was dialyzed against water, dried and the incorporation of glucose D-[¹⁴C] was quantified by liquid scintillation counting using 5% of the equal counts (50,000 cpm) were loaded on a 15% SDS-PAGE gel and separated by electrophoresis. Lipoglycans were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film).

PPM Synthase Assay

Membranes were prepared as described previously [37]. Briefly, cells were grown to mid-log phase, harvested, washed with PBS and stored at -20 °C. Cells were washed and resuspended in buffer A containing 50 mM MOPS (adjusted to pH 7.9 with KOH), 5 mM β-mercaptoethanol and 10 mM MgCl₂ [aq] at 4 °C and subjected to sonication for a total time of 10 min using 60 s pulses and 90 s cooling intervals. The preparations were centrifuged at 27000 x g for 25 min at 4 °C and the membranes were obtained by further centrifugation of the supernatant at 100,000 x g for 1 h at 4 °C. Membranes were resuspended in 1 ml of buffer A and concentration was determined using the BCA Protein Assay Reagent Kit (Thermo Scientific). Reaction mixtures for assessing [¹⁴C]Man incorporation consisted of 6.25 µCi GDP[Man-¹⁴C] (262 mCi/mmol; Perkin Elmer), 100 µM ATP, 10 mM MgCl₂ [aq], 100 µM dithiothreitol, 20 mM NaF [aq] and membrane preparations corresponding to 50–400 µg protein in a final volume of 100 µl. Decaprenyl monophosphate was added to the reaction mixtures at a final concentration of 125 µM. The reaction mixtures were then incubated at 37 °C for 30 min. The enzymic reactions were terminated by the addition CHCl₃/CH₃OH/0.8 M NaOH [aq] (10:10:3 by vol.) (6 ml/100 µl) followed by further incubation at 55 °C for 20 min. The mixtures were then allowed to cool; 2.625 ml of CHCl₃ and 1.125 ml of water were added. The mixture was vortexed and centrifuged and the upper aqueous phase discarded. The organic phase was washed three times with 2 ml of CHCl₃:CH₃OH:H₂O (3:47:48 by vol.), dried to yield an organic fraction containing PPMs. These were dried in a scintillation vial before scintillation counting using 5 ml of EcoScint A (National Diagnostics) [26].

Results and Discussion

Essentiality of MSMEG3859 in M. smegmatis

Due to its role in the biosynthesis of LM/LAM, Ms-ppm1 was considered to be an essential gene. We constructed a knockout phage phΔMsppm designed to replace MSMEG3859-MSMEG3860 in M. smegmatis with a hygromycin resistance cassette; we were however unable to generate a null mutant due to the failure to yield any transductants. In contrast, we were able to generate a MSMEG3859-MSMEG3860 null mutant by transducing a merodiploid strain containing a second, inducible copy of MSMEG3859-MSMEG3860 by CESTET (Conditional Expression Specialized Transduction Essentiality Test) [33]; suggesting that one or both genes were essential in M. smegmatis. As MSMEG3859 was shown to be sufficient for the enzymatic generation of PPM in vitro, we reasoned that the PPM synthase-encoding MSMEG3859, but not the membrane segment-encoding MSMEG3860, was an essential gene. To address this, we used CESTET again to test the essentiality of MSMEG3859 in M. smegmatis. First, a merodiploid strain was constructed by introducing a second copy of MSMEG3859 cloned into pTIC6a, an integrative plasmid driving expression via a tetracycline inducible promoter [30]. Expression of this recombinant copy of MSMEG3859 can be induced by adding anhydrotetracycline (ATc) to the growth medium. Following transduction with knockout phage phΔMsppm, we were able to generate knockout mutants only when transductants were selected on plates containing hygromycin and ATc suggesting that MSMEG3859 was essential in M. smegmatis. One such conditional mutant, designated ΔMsppm was used for further analysis. Subsequent passages of the ΔMsppm mutant in medium without the inducer ATc resulted in loss of viability indicating that expression of the pTIC6a-driven copy of MSMEG3859 was required for cell growth, confirming the essentiality of MSMEG3859 (Figure 2).

The ability to generate an MSMEG3859-MSMEG3860 double mutant in a MSMEG3859 merodiploid strain indicated that while the PPM synthase-encoding MSMEG3859 was essential, MSMEG3860 (which encodes a six transmembrane section-containing membrane-anchored protein) was not essential. This

![Figure 2. Essentiality of MSMEG3859 in M. smegmatis mc²155.](https://example.com/figure2.png)

Growth of the ΔMsppm conditional mutant on Tryptic Soy Agar with or without the inducer anhydrotetracycline (ATc). Ten microliters of 10-fold serial dilutions of cultures were spotted on the agar plates and incubated for 3 days at 37 °C. doi:10.1371/journal.pone.0048211.g002
correlated with earlier reports that MSMEG3859 was sufficient for PPM synthase activity [26,27].

**Loss of MSMEG3859 Results in Alteration of PIMs and Cessation of LM Biosynthesis**

As Ac1/Ac2PIM4 is at the branch point for the biosynthesis of higher PIMs (Ac1/Ac2PIM6) and LM/LAM biosynthesis, the conditional ΔMsPpm mutant could be used to determine whether loss of MSMEG3859-encoded PPM activity affected the biosynthesis of these molecules. The mutant was grown for 36 hours in media in the presence, or absence, of ATc labelled with [14C]-glucose and subjected to lipid extractions. Cultures grown in ATc-containing media showed all PIM intermediates present. In contrast, cultures of the conditional mutant grown in the absence of ATc showed increasing amounts of PI accompanied by decreasing levels of Ac1/Ac2PIM6 (Figure 3). The loss of Ac1/ Ac2PIM6 and accumulation of PIMx precursors suggested that MSMEG3859 is required for the synthesis of higher order PIMs, particularly Ac1/ Ac2PIM6. Conditional depletion of PPM synthase activity in the ΔMsPpm mutant should also affect the biosynthesis of lipoglycans and cultures grown in the absence of ATc did show diminished levels of [14C]LM (Figure 4). However, we did not see major differences in [14C]LM levels. The loss of [14C]LM but presence of [14C]LAM from endogenous PPM generated prior to conditional depletion. Thus, the observed LAM in the depleted cells was likely from residual LM, rather than newly synthesised LM, as observed through dual [14C]/[3H] dual labelling experiments by Besra et al. [37]. These data suggest that MSMEG3859, which encodes the equivalent of *M. tuberculosis* Ppm1/D2, is solely responsible for the PPM synthase activity required for the generation of higher order PIMs and LM/LAM.

![Figure 3. 2D-TLC analysis of [14C]-labeled polar lipids from the ΔMsPpm conditional mutant.](image)

![Figure 4. Lipoglycan profile of the ΔMsPpm conditional mutant.](image)

*Ppm1 Is Essential in Mycobacteria*
Effects of Loss of MSMEG3859 on Membrane-associated PPM Synthase Activity

Membrane preparations from cultures of the ΔMsPpm mutant, grown in the presence and in the absence of ATc, were used to assay membrane-associated PPM synthase activity. A PPM synthase assay was used to analyse PPM activity in the membranes using GDP-[14C] Man and polyrenol phosphate as substrate. This assay was used to assess the effects of depletion of MSMEG3859 function on the ability of the membrane preparations to catalyse the formation of PPM (C50-P-Man) donors via the incorporation of radioactive mannose into polyrenol substrate through pooled organic extracts containing PPMs [26]. While membrane preparations from cultures grown in the presence of ATc were able to catalyse incorporation of [14C] Man into polyrenols, those from cultures grown in the absence of ATc displayed poor PPM synthase activity (Figure 5). This co-relation between depleted MSMEG3859 function and low PPM synthase activity confirmed that MSMEG3859 was the key synthase required to catalyze the production of PPM donors in M. smegmatis.

Potential Ability of the Alternative M. tuberculosis PPM Synthases to Rescue Viability and Restore Wild-type Phenotype in the ΔMsPpm Mutant

In contrast to M. smegmatis, M. tuberculosis encodes two other membrane-associated glycosyltransferases, Rv3779 and Rv3631 (PpgS), which have been suggested to have putative roles as PPM synthases in M. tuberculosis [30]. As mentioned above, homologues of neither are not present in M. smegmatis. The ΔMsPpm conditional mutant thus offered us an opportunity to assess the possible roles of Rv3779 and PpgS as alternative PPM synthases.

Each gene could be functionally analyzed in vivo by testing the ability of the ΔMsPpm conditional mutant transformed with a plasmid-borne copy of either Rv3779 or PpgS to rescue Ac1/Ac2PIM6 biosynthesis when cultured in medium devoid of ATc. As expected, Ac1/Ac2PIM6 biosynthesis was not affected in non-ATc cultures of ΔMsPpm containing plasmid clones of either MSMEG3859 [Figure 6]. We then tested the ability of the M. tuberculosis equivalent, Mt-ppm1/D2 to rescue the loss of MSMEG3859 function in the same manner and found Ac1/Ac2PIM6 levels unaffected in the recombinant strains [Figure 6] indicating that Mt-ppm1/D2 was functional in M. smegmatis. In contrast, however, Rv3779 was unable to rescue Ac1/Ac2PIM6 biosynthesis in the conditional mutant when grown in the absence of ATc, leading to a loss of Ac1/Ac2PIM6 and a noticeable accumulation of intermediary Ac1/Ac2PIMxs (Figure 6). Thus while Rv3779 was shown to have PPM synthase activity in vitro [29], it surprisingly failed to substitute for loss of MSMEG3859 in the conditional ΔMsPpm mutant. Thus, in contrast to its in vitro activity, it is unlikely that Rv3779 functions in vivo as a PPM synthase. Instead, its predominant role in M. tuberculosis seems to be the utilisation of polyrenyl-P-D-GalNAc as a donor for the biosynthesis of galactosamine-modified AG [30].

As mentioned above, while PpgS is involved in the generation of polyrenyl-phospho-N-acetylgalactosamine (polyrenyl-P-GalNAc) from polyrenyl-P and UDP-GalNAc, it could be envisaged as a secondary, potential PPM synthase. Its syntenic association with the small integral membrane protein Rv3662 is analogous to MSMEG3859-MSMEG3860, and Mt-Ppm-1/D2-Mt-Ppm-1/D1. However, ppgS also failed to compensate for the loss of MSMEG3859 in the conditional mutant (Figure 6), and thus is unlikely to function as a PPM synthase in vivo.

Together, these results indicate that ppm1 is the gene encoding the sole PPM synthase in M. tuberculosis capable of generating the mannos donor for subsequent higher order PIMs and LM/LAM biosynthesis.

Figure 5. PPM synthase activity in membranes of the ΔMsPpm conditional mutant. Graph shows the transfer of [14C]-mannose from GDP[Man-14C(U)] (262 mCi/mmol; Perkin Elmer) to polyrenol-phosphate in membrane extracts prepared from cultures grown with or without anhydrotetracycline (ATc).
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CH3OH/H2O (60:30:6, v/v/v) in the first direction and CHCl3:CH3OOH:CH3OH:H2O (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were applied to Silica Gel 60 F254 (Merck 5554) aluminium-backed TLC plates and developed using solvent system E for polar lipids: CHCl3/CH3OH/H2O (60:30:6, v/v/v) in the first direction and CHCl3:CH3OOH:CH3OH:H2O (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film). PIM intermediates are shown by arrows (x = 3–5) and the dotted circle indicates the position of the higher PIMs on the TLC plates.

Figure 6. Complementation of the ΔMsPpm conditional mutant. 2D-TLC analysis of [14C]-labelled polar lipids from the ΔMsPpm conditional mutant complemented with (A) pAKR-MsMSEG3859 (B) pAKR-Mt-ppm1/D2 (C) pAKR-Rv3779 (D) pAKR-ppgS. Equal counts of polar lipid extracts (50,000 cpm) were applied to Silica Gel 60 F254 (Merck 5554) aluminium-backed TLC plates and developed using solvent system E for polar lipids: CHCl3/CH3OH/H2O (60:30:6, v/v/v) in the first direction and CHCl3:CH3OOH:CH3OH:H2O (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film). PIM intermediates are shown by arrows (x = 3–5) and the dotted circle indicates the position of the higher PIMs on the TLC plates.

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Author Contributions
Conceived and designed the experiments: AR AS SG. Analyzed the data: AR SG LRC AB GSB.

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