SUPPLEMENTAL MATERIAL

NMR analysis of purified mannolipids 1 and 2 - The complete chemical structures of native PIM_2 and PIM_6 in their deacylated and acylated forms have been reported (main text references 19, 20, 21). It was clearly established that in the case of PIM_2, the mannosyl units were attached to positions 2 and 6 of the *myo*-Ins (*myo*-Inositol) ring of PI. Taking advantage of this information, a combination of 1D and 2D-NMR was then used to determine the position at which the Man*p* residues were attached to *myo*-Ins in mannolipids 1 and 2 (Fig. 2 and Fig. 3S, 4S, 5S and 6S).

While the ^{1}H and ^{13}C peaks of the glycerol moiety and lipid chains of mannolipids 1 and 2 appeared at identical chemical shifts in both mannolipids as well as in PI, the ^{1}H and ^{13}C chemical shifts of the *myo*-Ins moiety were different in mannolipids 1 and 2 reflecting different attachment sites of the Man*p* residue. In the ^{1}H NMR spectra of mannolipid 1, the chemical shifts of the H-1, H-3, H-4, H-5 and H-6 protons of *myo*-Ins moiety were similar to that of PI. However, the chemical shift of the H-2 proton showed a higher value (4.296 ppm) when compared to the corresponding peak in PI (4.173 ppm) (Fig. 3S). In addition, the COSY spectra of mannolipid 1 revealed that both the H-1 and H-3 protons of *myo*-Ins showed correlation to the peak at 4.296 ppm. Therefore, this peak was undoubtedly assigned to the H-2 proton of the *myo*-Ins moiety.

It has been demonstrated previously that the α-anomer protons usually resonate downfield compared to the β-anomers (31). The vicinal coupling constant between the anomeric H-1 and the H-2 indicates the relative orientation of the two protons in the sugar ring. In particular, the coupling constant (J) of α-anomer protons has been reported to be < 2Hz (31). Based on its chemical shift value and J coupling (1.60 Hz), the anomeric proton of Man*p* (5.14 ppm) in mannolipid 1 has been assigned to the α-anomer. This assignment is in accordance with the reported native chemical structure of PIM_2 and its acylated forms (19, 20, 21). Using the α-anomeric proton as a starting point, the complete ^{1}H chemical shifts of the Man*p* ring in mannolipid 1 have been deduced using a

combination of COSY and TOCSY NMR. (Fig. 4S). Then, the corresponding ^{13}C chemical shifts were deduced by using the ^{1}H-^{13}C HSQC spectra (Fig. 5S). In the 2D TOCSY spectrum of mannolipid 1, the α-anomeric proton of Man*p* at 5.14 ppm showed long range correlation to H-2 of *myo*-Ins at 4.296 ppm (Fig. 4S). Similarly, in the ^{1}H-^{13}C HMBC spectrum of mannolipid 1, the α-anomeric proton of Man*p* at 5.14 ppm showed long range correlation to the ^{13}C peak C-2 of *myo*-Ins at 79.14 ppm (Fig. 5S). It is also noteworthy that, in mannolipid 1, the ^{1}H and ^{13}C peaks corresponding to position-2 of *myo*-Ins were downfield shifted by 0.12 and 7.02 ppm, respectively, as compared to that of PI. Altogether, the experimental data clearly demonstrated that mannolipid 1 corresponds to α–2-linked PIM_1.

In the ^{1}H NMR spectrum of mannolipid 2, the chemical shift of the H-1, H-2, H-3, H-4 and H-5 protons of *myo*-Ins moiety were similar to that of PI. However the chemical shift of H-6 showed a higher value (3.94 ppm) when compared to the corresponding H-6 proton in PI (3.793 ppm) (Fig. 6S). The entire ^{1}H chemical shift of mannolipid 2 was deduced in a similar manner as explained above using the combination of COSY and TOCSY NMR. Due to limiting quantities of mannolipid 2, 2D heteronuclear experiments were not performed in this case. In the ^{1}H spectra of mannolipid 2, the α-anomeric proton appeared at 5.072 ppm (J = 1.6Hz), which showed long-range correlation (TOCSY) to the H-6 proton of *myo*-Ins at 3.94 ppm. From these combined results, mannolipid 2 was assigned to α–6-linked PIM_1.

NMR analysis of purified mannolipids 5 – In the ^{1}H spectra of mannolipid 5, the chemical shift of H-2, H-3, H-4 and H-5 were similar to that of Man*p* residues linked to positions 2 and 6 of *myo*-Ins (Fig. 8SA and 8SB). However, two sets of H-6 (AB multiplets) proton signals were observed as evidenced by the correlation of H-5 to H-6 in the COSY spectrum. One set of H-6 protons appeared at 3.74 and 3.83 ppm and another set appeared at 4.05 and 4.10 ppm. Further, 2D ^{1}H-^{13}C HMBC showed long-range correlation from H-6 protons at 4.05 and 4.10 ppm to a carboxylic ^{13}C peak corresponding to an esterified acyl chain, which confirmed the acylation of the Man*p* residue at position 6 (Fig 8SC).

REFERENCES

31. Duus, J., Gotfredsen, C. H., Bock, K. (2000) *Chem Rev.* **100**, 4589-614.

SUPPLEMENTAL FIGURES

Fig. 1S. Purification of a recombinant form of *Ms*PimB'. 2 μg of purified recombinants forms *Ms*PimA and *Ms*PimB were run on a SDS-PAGE and stained with SimplyBlue^{TM} SafeStain (Invitrogen).

Fig. 2S. MALDI-TOF/MS analysis of the purified mannolipids 1, 2, 3, 4 and 5.

Fig. 3S. NMR analysis of purified mannolipids 1 and 2. *A-B.* ^{1}H NMR spectrum of mannolipid 1 with complete assignment of peaks.

Fig. 4S. NMR analysis of purified mannolipids 1 and 2. *A.* ^{1}H-^{1}H TOCSY spectrum of mannolipid 1 showing the complete assignment of peaks. *B.* Selected region of the ^{1}H-^{1}H TOCSY spectrum showing diagnostic correlation from the α-anomeric proton of Man*p* (5.14 ppm) to the H-2 proton of *myo*-Ins (4.296 ppm).

Fig. 5S. NMR analysis of purified mannolipids 1 and 2. *A.* ^{1}H-^{13}C HSQC spectrum of mannolipid 1 with assignments of cross peaks. *B.* Selected region of the ^{1}H-^{13}C HMBC spectrum of mannolipid 1 showing the diagnostic correlation for the α-anomeric proton of Man*p* (5.14 ppm) to the C-2 of *myo*-Ins (79.14 ppm).

Fig. 6S. NMR analysis of purified mannolipids 1 and 2. Selected regions of the ^{1}H NMR spectrum of mannolipid 1 (A), mannolipid 2 (B) and PI (C). Compared to PI, one can clearly observe the downfield shift of the H-2 and H-6 protons of *myo*-Ins upon Man*p* linkage in mannolipid 1 and 2, respectively.

Fig. 7S. Inactivation of *Ms*PimA and *Ms*PimB' at 60ºC. TLC autoradiograph of enzymatic reactions performed with purified recombinant *Ms*PimA and *Ms*PimB' pre-incubated at 37ºC and 60ºC.

Fig. 8S. NMR analysis of purified mannolipid 5. *A-B.* ^{1}H NMR spectrum of mannolipid 5 with complete assignment of peaks. *C*. Selected region of the ^{1}H-^{13}C HMBC spectrum of mannolipid 5 showing diagnostic correlation for the H-6 proton of Man*p* (4.055 and 4.095 ppm) to the carboxylic carbon of the acyl group (174.4 ppm). This data clearly indicates that the acyl group is linked to position 6 of Man*p*.

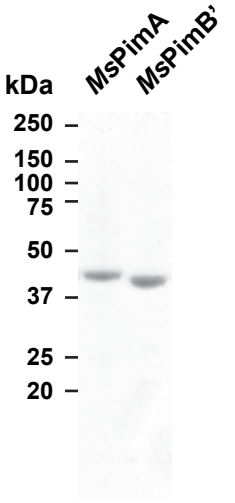


Figure 1S

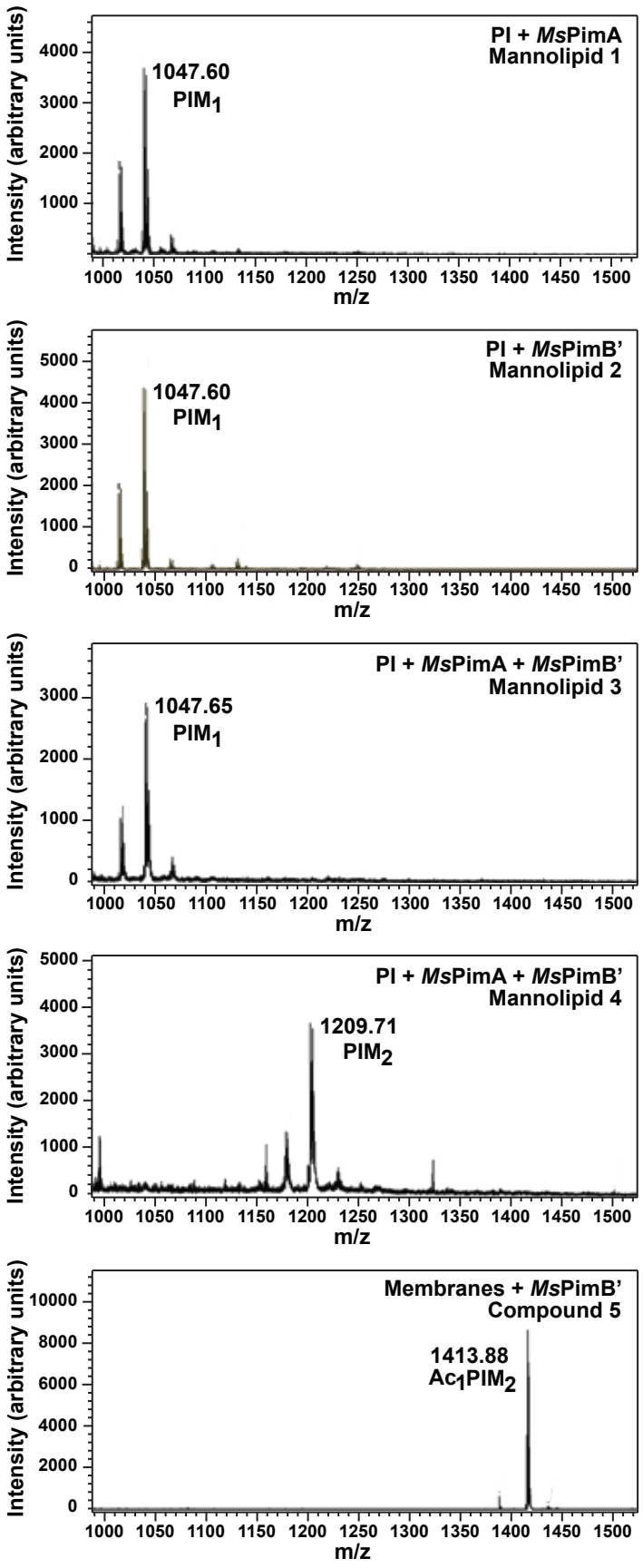

PI + MsPimA
Mannolipid 1
1047.60
PIM1
PI + MsPimB'
Mannolipid 2
1047.60
PIM1
PI + MsPimA + MsPimB'
Mannolipid 3
1047.65
PIM1
PI + MsPimA + MsPimB'
Mannolipid 4
1209.71
PIM2
Membranes + MsPimB'
Compound 5
1413.88
Ac1PIM2
Intensity (arbitrary units)
m/z

Figure 2S

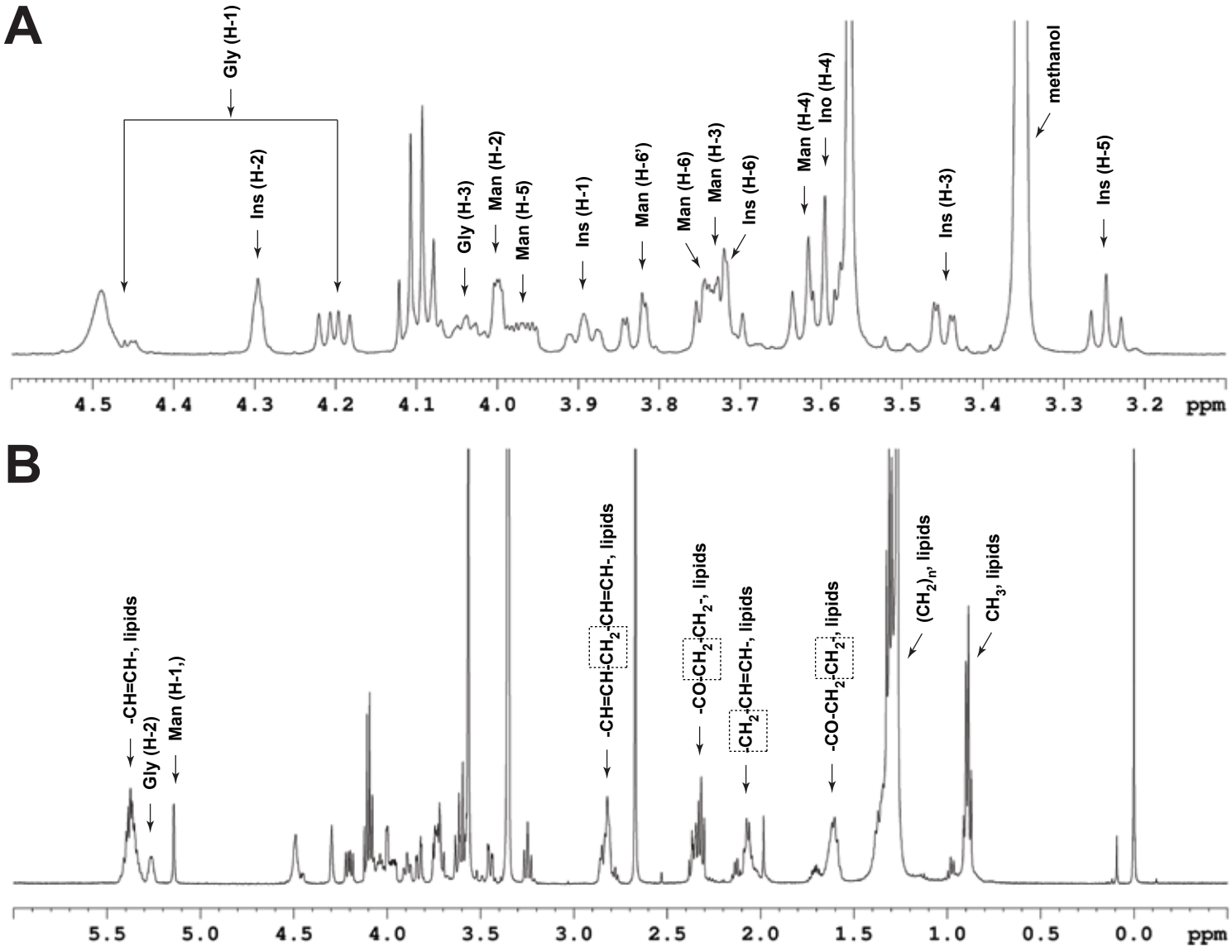
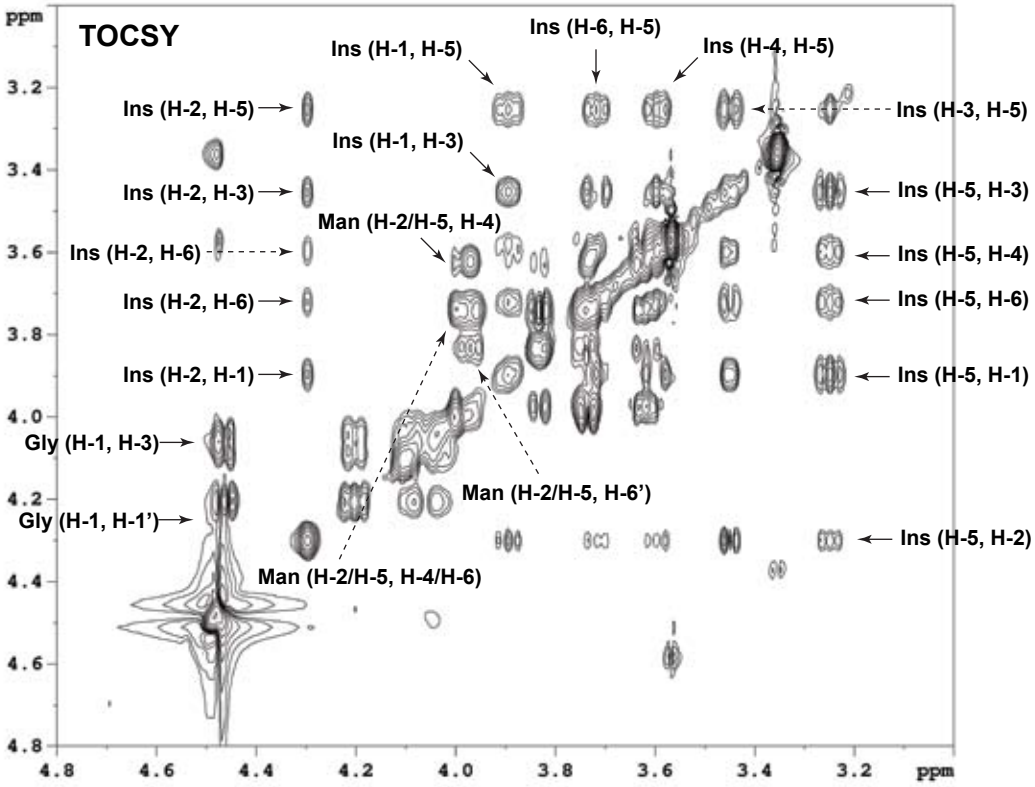

A
Gly (H-1)
Ins (H-2)
Gly (H-3)
Man (H-2)
Man (H-5)
Ins (H-1)
Man (H-6')
Man (H-6)
Man (H-3)
Ins (H-6)
Man (H-4)
Ino (H-4)
methanol
Ins (H-3)
Ins (H-5)
4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 ppm
B
-CH=CH-, lipids
Gly (H-2)
Man (H-1,)
-CH=CH-CH2-CH=CH-, lipids
-CO-CH2-CH2-, lipids
CH2-CH=CH-, lipids
-CO-CH2-CH2-, lipids
(CH2)n, lipids
CH3, lipids
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm

Figure 3S

A


TOCSY
ppm
Ins (H-1, H-5)
Ins (H-6, H-5)
Ins (H-4, H-5)
Ins (H-2, H-5)
Ins (H-3, H-5)
Ins (H-1, H-3)
Ins (H-2, H-3)
Ins (H-5, H-3)
Man (H-2/H-5, H-4)
Ins (H-2, H-6)
Ins (H-5, H-4)
Ins (H-2, H-6)
Ins (H-5, H-6)
Ins (H-2, H-1)
Ins (H-5, H-1)
Gly (H-1, H-3)
Man (H-2/H-5, H-6')
Gly (H-1, H-1')
Ins (H-5, H-2)
Man (H-2/H-5, H-4/H-6)
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
ppm

B

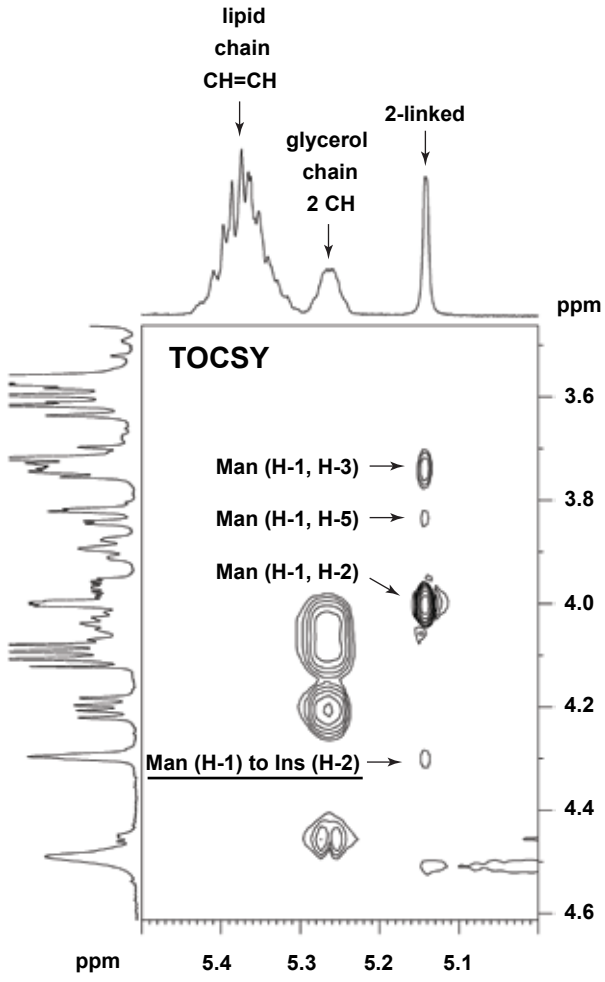

lipid
chain
CH=CH
2-linked
glycerol
chain
2 CH
ppm
TOCSY
Man (H-1, H-3)
Man (H-1, H-5)
Man (H-1, H-2)
Man (H-1) to Ins (H-2)
3.6
3.8
4.0
4.2
4.4
4.6
ppm
5.4
5.3
5.2
5.1

Figure 4S

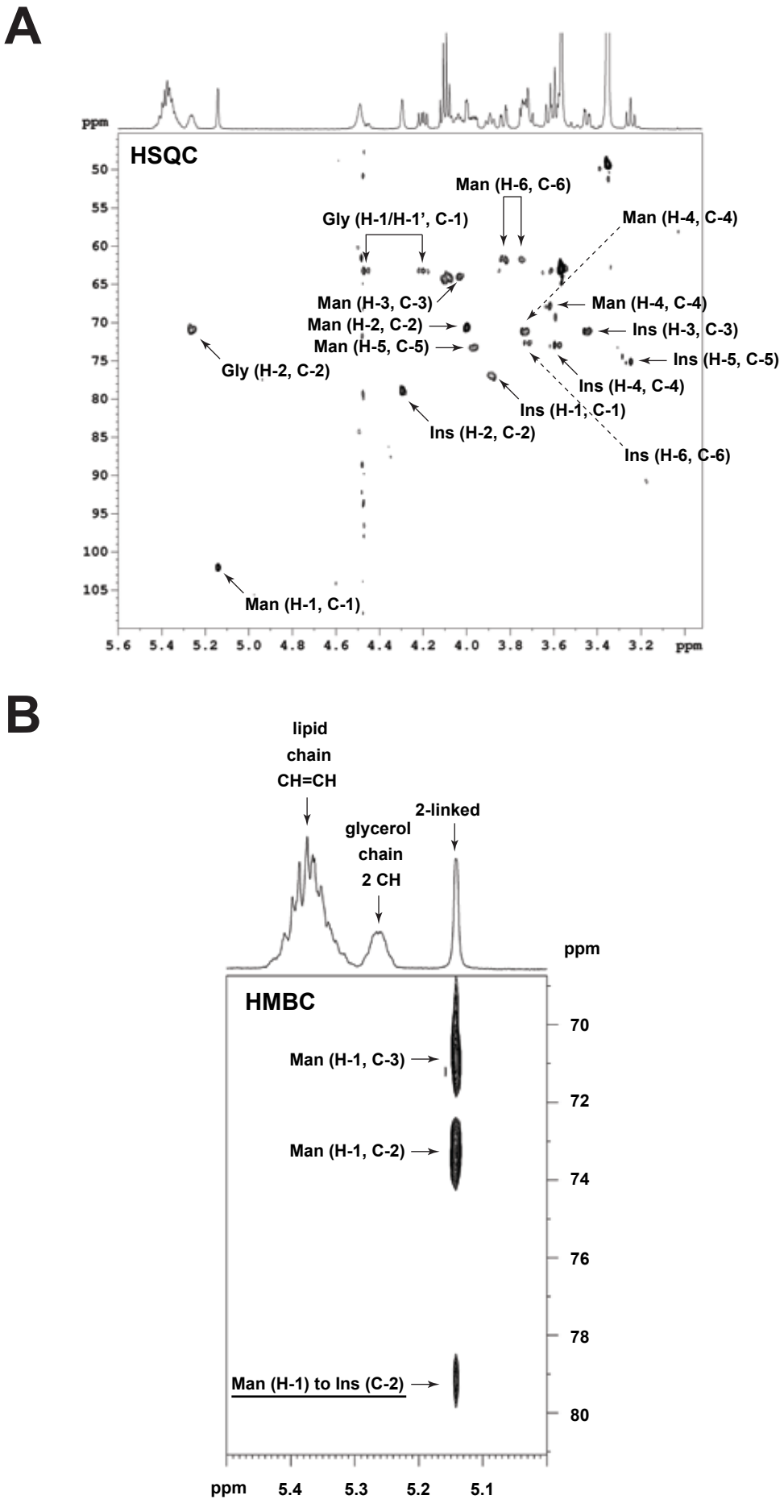

A
HSQC
ppm
Man (H-6, C-6)
Gly (H-1/H-1', C-1)
Man (H-4, C-4)
Man (H-3, C-3)
Man (H-4, C-4)
Man (H-2, C-2)
Ins (H-3, C-3)
Man (H-5, C-5)
Ins (H-5, C-5)
Gly (H-2, C-2)
Ins (H-4, C-4)
Ins (H-1, C-1)
Ins (H-2, C-2)
Ins (H-6, C-6)
Man (H-1, C-1)
B
lipid
chain
CH=CH
2-linked
glycerol
chain
2 CH
ppm
HMBC
Man (H-1, C-3)
Man (H-1, C-2)
Man (H-1) to Ins (C-2)
ppm

Figure 5S

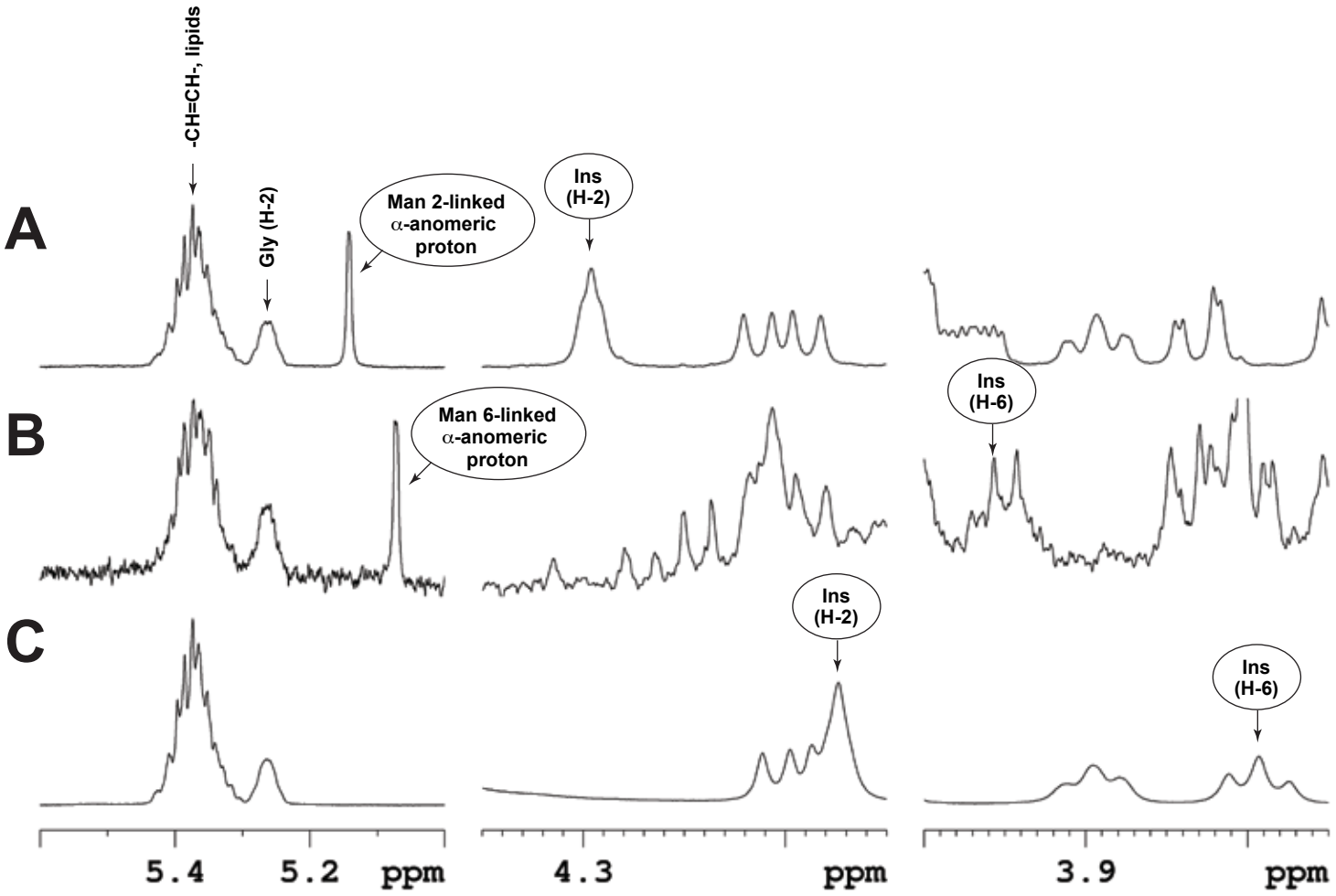

A
B
C
-CH=CH-, lipids
Gly (H-2)
Man 2-linked
α-anomeric
proton
Ins
(H-2)
Ins
(H-6)
Man 6-linked
α-anomeric
proton
Ins
(H-2)
Ins
(H-6)
5.4
5.2
ppm
4.3
ppm
3.9
ppm

Figure 6S

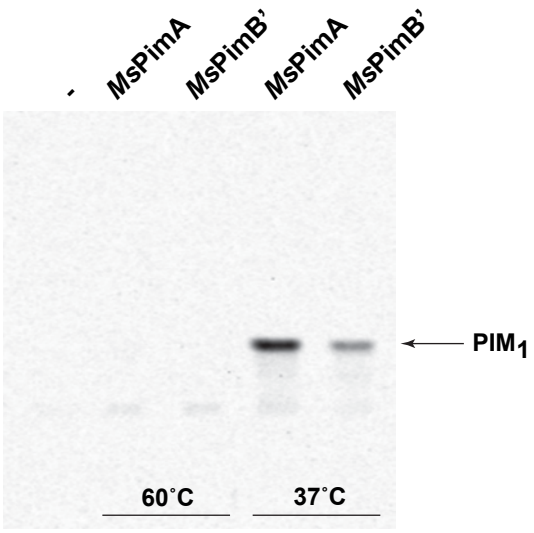


Figure 7S

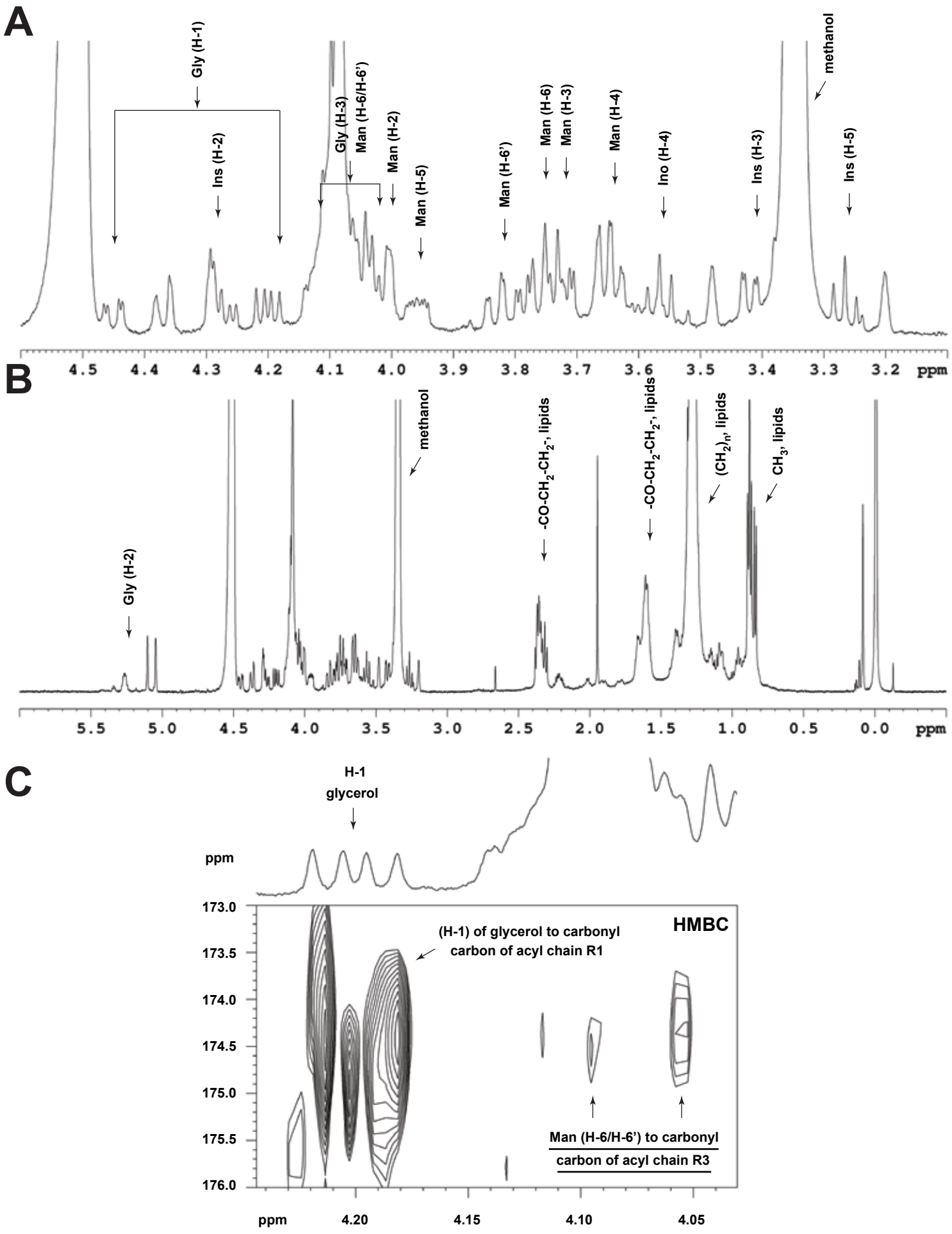

A
Gly (H-1)
Ins (H-2)
Gly (H-3)
Man (H-6/H-6')
Man (H-2)
Man (H-5)
Man (H-6')
Man (H-6)
Man (H-3)
Man (H-4)
Ino (H-4)
Ins (H-3)
methanol
Ins (H-5)
4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 ppm
B
Gly (H-2)
methanol
-CO-CH2-CH2-, lipids
-CO-CH2-CH2-, lipids
(CH2)n, lipids
CH3, lipids
5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 ppm
C
H-1
glycerol
ppm
173.0
173.5
174.0
174.5
175.0
175.5
176.0
HMBC
(H-1) of glycerol to carbonyl
carbon of acyl chain R1
Man (H-6/H-6') to carbonyl
carbon of acyl chain R3
ppm 4.20 4.15 4.10 4.05

Figure 8S