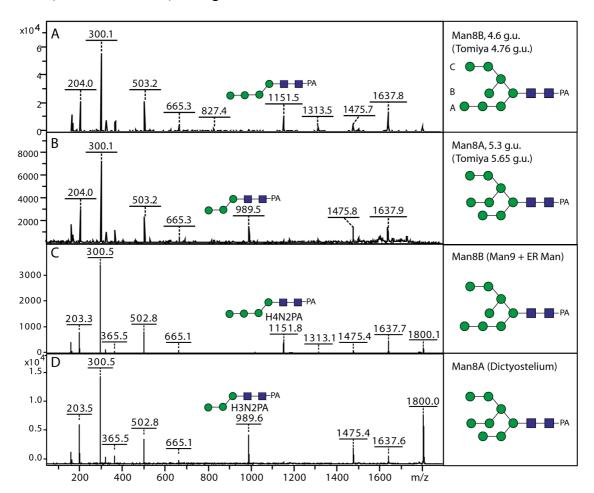
## COMPARISON OF RP-HPLC MODES TO ANALYSE THE N-GLYCOME OF THE FREE-LIVING NEMATODE PRISTIONCHUS PACIFICUS

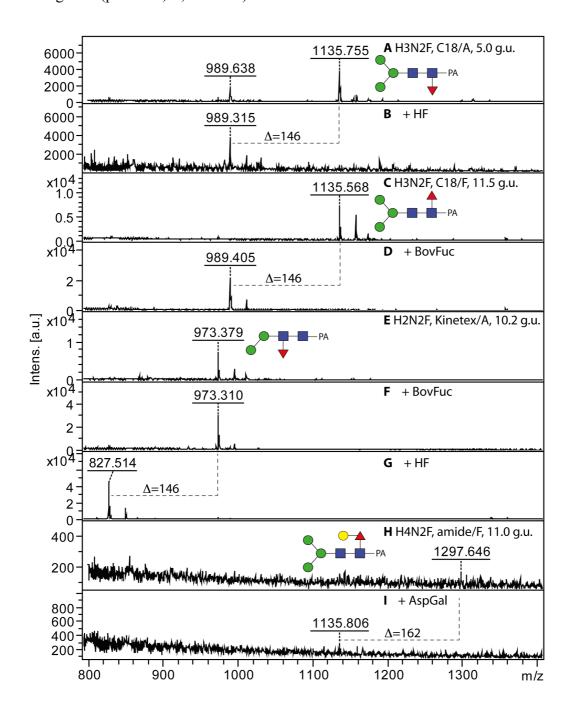
Shi Yan, Iain B. H. Wilson and Katharina Paschinger

Supplementary Data

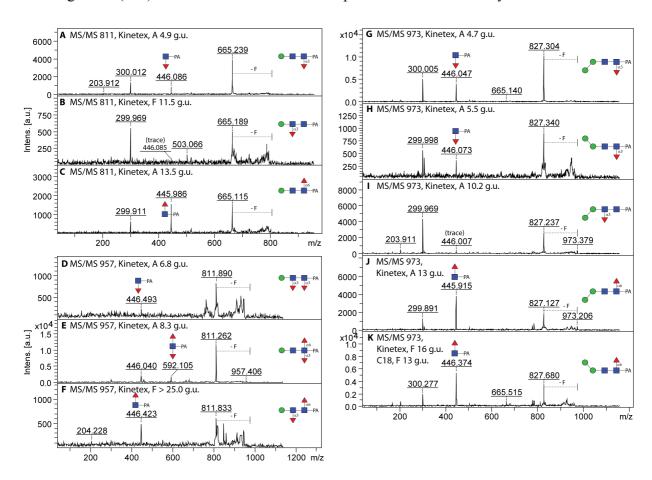
Supplementary Figure 1. Fragmentation patterns of Man<sub>8</sub>GlcNAc<sub>2</sub> isomers: The MALDI-TOF MS/MS spectra for the two isomers of Man<sub>8</sub>GlcNAc<sub>2</sub> found in different RP-HPLC fractions of the pyridylamino-labelled pool of the *Pristionchus* PNGase F released N-glycans (A, B) are compared to a standard Man<sub>8</sub>B (generated by the action of *Caenorhabditis elegans* ER mannosidase on commercial Man<sub>9</sub>GlcNAc<sub>2</sub>; C) and the Man<sub>8</sub>A isomer isolated from *Dictyostelium discoideum* (D). The latter two spectra are also presented in Supplementary Figure 4 of Ref. 32. The different branches (A, B and C) of the Man<sub>8</sub> structure are also annotated as are the RP-HPLC glucose unit values given by Tomiya *et al* in their 1991 paper (Ref. 41). Note the absence of the m/z 1151 fragment from Man<sub>8</sub>A, which instead has an m/z 989 fragment; this pattern is interpreted as indicating loss of the  $\alpha$ 1,6-antenna (B and C branches) during MS/MS.



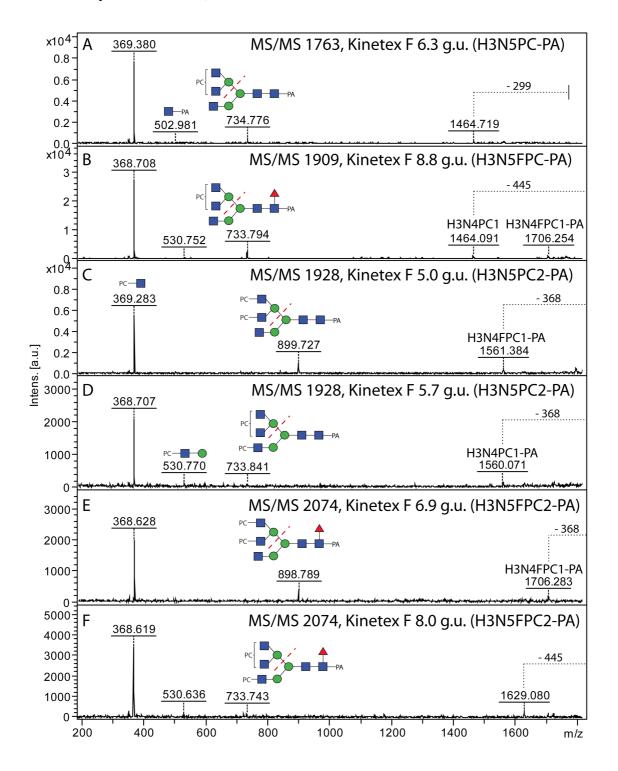
**Supplementary Figure 2.** Exoglycosidase and chemical treatments of monofucosylated N-glycans: The effect of hydrofluoric acid (B and G), bovine α-fucosidase (D and E) or Aspergillus β-galactosidase (D on four different monofucosylated pyridylamino-labelled Pristionchus N-glycans (Hex<sub>2-4</sub>GlcNAc<sub>2</sub>Fuc<sub>1</sub>) as shown by MALDI-TOF MS. Note that the α1,3-fucose on the proximal or distal GlcNAc residues is sensitive to HF, whereas α1,6-fucose on the proximal GlcNAc is sensitive to bovine α-fucosidase (the relevant panel D is the non-magnified version of Figure 6E) and the galactose attached to core fucose is lost upon Aspergillus galactosidase treatment; loss of the relevant m/z 446 or 608 core fragments was confirmed. Other data on hydrofluoric acid, fucosidase and galactosidase sensitivities of related structures are shown in Figure 5 and 6 of the main text. The annotated MS/MS spectra of the original glycans A (m/z 1135), C (m/z 1135), E (m/z 973) and E (m/z 1297) are shown in Figure 4 (panels A, B, C and G) of the main text.



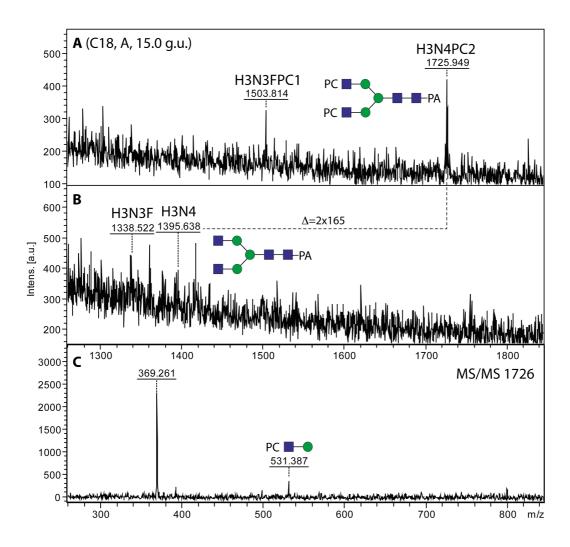
Supplementary Figure 3. Fragmentation patterns of fucosylated pyridylamino-labelled N-glycans: MS/MS spectra for the three differently-eluting isomers of Man<sub>1</sub>GlcNAc<sub>2</sub>Fuc<sub>1</sub> (m/z 811; A-C), three isomers of Man<sub>1</sub>GlcNAc<sub>2</sub>Fuc<sub>2</sub> (m/z 957; D-F) and five isomers of Man<sub>2</sub>GlcNAc<sub>2</sub>Fuc<sub>1</sub> (m/z 973; G-K) from *Pristionchus* (panel I is also as shown in Figure 4C of the main text). As mentioned in the main text:  $\alpha$ 1,6-fucosylation of the reducing-terminal proximal GlcNAc results in strong m/z 446 fragments (C, F, J, K),  $\alpha$ 1,3-fucosylation of the proximal GlcNAc results in m/z 300 and 446 fragments of approximately equal intensity (A, G, H), difucosylation of the proximal GlcNAc results in m/z 446 and 592 fragments of approximately equal intensity (E) and  $\alpha$ 1,3-fucosylation of the distal (second core) GlcNAc is often associated with weak (trace) m/z 446 fragments resulting from a low level of fucose rearrangement (B, I). Loss of one fucose from the parent ion is indicated by 'F'.



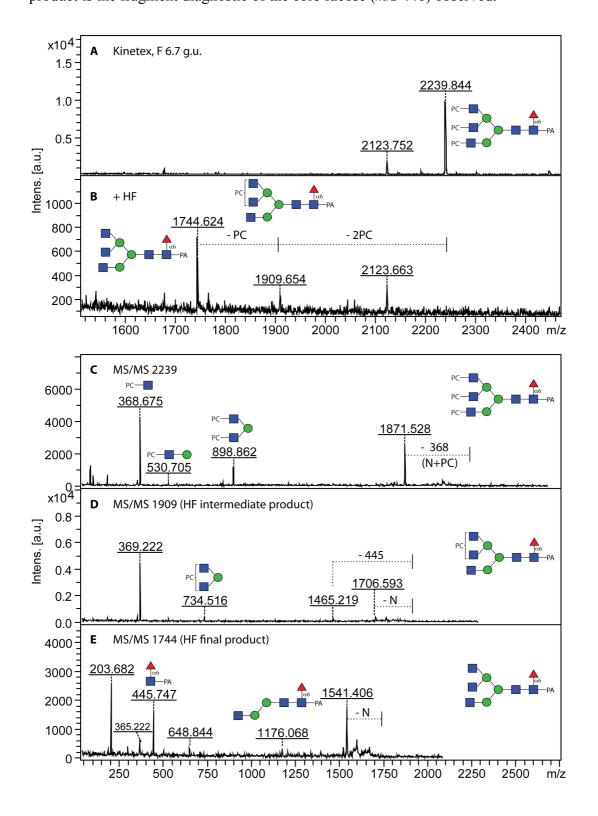
**Supplementary Figure 4. MALDI-TOF MS/MS analysis of triantennary N-glycans modified with one or two phosphorylcholine residues.** Further examples of the fragmentation patterns of phosphorylcholine-modified pyridylamino-labelled N-glycans from *Pristionchus*, including two isomers each of Hex<sub>3</sub>HexNAc<sub>5</sub>Fuc<sub>0-1</sub>PC<sub>2</sub>. The overall predicted structures are shown with an indication of the predicted fragmentation position for the diagnostic *m/z* 734 and 899 ions (panel A is a further version of Figure 8E in the main text). Losses of either the core GlcNAc-PA or GlcNAcFuc-PA or an antennal GlcNAc-PC is indicated by the loss of 299, 445 or 368.



**Supplementary Figure 5. MALDI-TOF MS and MS/MS of Hex<sub>3</sub>HexNAc<sub>4</sub>PC<sub>2</sub>:** A biantennary N-glycan predicted to carry two phosphorylcholine residues (*m/z* 1725; A) was treated with hydrofluoric acid prior to MALDI-TOF MS (B); the *m/z* 1503 glycan is carried over from the neighbouring fraction (for its analysis see Figure 9 D-F of the main text). The products are primarily present as sodiated adducts, but the protonated ions are annotated. MS/MS of the original glycan (C) shows phosphorylcholine-containing fragments of *m/z* 369 and 531 GlcNAc (Hex<sub>0-1</sub>HexNAc<sub>1</sub>PC<sub>1</sub>) compatible with phosphorylcholine modification of non-reducing terminal.



## Supplementary Figure 6. Chemical treatment of a phosphorylcholine-modified N-glycan: A triantennary core fucosylated N-glycan predicted to carry three phosphorylcholine residues (A) was treated with hydrofluoric acid prior to MALDI-TOF MS (B). MS/MS of the original glycan (C) and of the intermediate (D) and final products (E) show the dominance of the phosphorylcholine-containing fragments (m/z 369, 530, 734 and 898); only with the final product is the fragment diagnostic of the core fucose (m/z 446) observed.



**Supplementary Table. Summary of evidence for the proposed structures of** *Pristionchus* **N-glycans.** An extended version of Table 1 (see main text) is presented with brief summaries of the evidence for the proposed structures referring also to relevant figures, supplementary figures and citations. Retention times for the three glucosylated N-glycans (Glc<sub>1-3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>; not shown for reasons of space in Table 1) are also included.

Structure	m/z	Retention time			Evidence
		RP C18	RP-amide	Kinetex	
	665.28	A/F 6.4	A/F 6.2	F 7.8	Based on biosynthetic pathways Tomiya, et al. (1991) Anal Biochem 193, 90; 6.60 g.u.
a3	811.35	A 4.3	A 4.3	A 4.9	Only found in PNGase A released pool. Key fragment: 446 [suppl. Fig 3A]
α3	811.35	A/F 7.7	F 7.5	A/F 11.5	Trace amount of 446 fragment due to rearrangement [suppl. Fig 3B]
a6	811.35	A/F 10.3	A/F 8.2	A/F 13.5	[Fig 3E and F] Key fragment: 446 [suppl. Fig 3C]
	827.34 (0M)	A/F 5.9	A/F 6.1	A/F 7.5	[Fig 3B, sensitive to α1,2/3 mannosidase] Tomiya, et al. (1991); 6.59 g.u.
	827.34 (M0)	A/F 7.7	A/F 7.2	A/F 9.5	Tomiya, et al. (1991); 7.40 g.u. Sensitive to α1,6 mannosidase
a3 a3	957.40	A 5.9	n.d.	A 6.8	Only found in PNGase A released pool. Key fragment: 446 [suppl. Fig 3D]
a6 a3	957.40	A 7.0	A 6.3	A 8.3	Only found in PNGase A released pool. Key fragment: 592 [suppl. Fig 3E] [also Fig 3I]
a3	957.40	n.d.	A 8.7	F > 25.0	Key fragment: 446 [suppl. Fig 3F]
α6 α3 Me	971.42	A 19.0	A 9.5	A >25	Only found in PNGase A released pool. Key fragments: 446 and 606 [Fig 5E and F, sensitive to bovine fucosidase and HF]
a <sub>3</sub>	973.40 (0MF <sup>3</sup> )	A 4.2	A 4.5	A 4.7	Only found in PNGase A released pool. Key fragment: 446 [suppl. Fig 3G]
a3	973.40 (M0F <sup>3</sup> )	A 5.0	A 5.0	A 5.5	Only found in PNGase A released pool. Key fragment: 446 [suppl. Fig 3H] Sensitive to $\alpha$ 1,6 mannosidase
a <sub>3</sub>	973.40	A/F 7.7	F 7.2	A/F 10.2	[Suppl. Fig 2 F and G, resistant to bovine fucosidase but sensitive to HF] Sensitive to α1,2/3 mannosidase Key fragment: trace 446, due to rearrangement [suppl. Fig 3I]
αδ	973.40 (0MF <sup>6</sup> )	A/F 9.8	A/F 8.2	A/F 13	Key fragment: 446 [suppl. Fig 3J] [Fig 3D, sensitive to α1,2/3 mannosidase] Sensitive to bovine fucosidase
Q6	973.40 (M0F <sup>6</sup> )	A/F 13.0	A/F 9.0	A/F 16	Key fragment: 446 [suppl. Fig 3K] Sensitive to bovine fucosidase

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	989.39 (MM)	A/F 7.2	A/F 7.2	A/F 9.5	Tomiya, et al. (1991); 7.39 g.u. [Fig 3J, sensitive to α1,2/3 mannosidase] Trimannosyl core structure as found in many invertebrates
	1030.42 (0Gn)	A/F 6.5	A/F 6.1	A/F 7.5	[Fig 7B, sensitive to FDL]
α3 α3	1119.46	A 5.2	A 5.4	A 6.8	Only found in PNGase A released pool. Key fragment: 446 Sensitive to $\alpha$ 1,2/3 mannosidase
06 03	1119.46	A 7.0	A 6.3	A 8.3	Only found in PNGase A released pool. [Fig 3J, sensitive to $\alpha$ 1,2/3 mannosidase] Key fragment: 592 [Fig 4D]
a3	1119.46	F 13.0	n.d.	F >25.0	Key fragments: 446 [Fig 4E] Sensitive to bovine fucosidase
	1135.45 (MMF <sup>3</sup> )	A 5.0	A 5.1	A 5.5	Only found in PNGase A released pool. Key fragment: 446 [Fig 4A] HF sensitive [Suppl. Fig. 2B]
ta6	1135.45 (MMF <sup>6</sup> )	A/F 11.5	A/F 9.5	A/F 16.0	Key fragment: 446 [Fig 4B] Tomiya, et al. (1988) 10.2 g.u. [Fig 6D and E, sensitive to α1,2/3 mannosidase and bovine fucosidase; also Suppl. Fig. 2D]
μα6	1176.48	n.d.	F 8.2	F 13.0	Based on structure of m/z 1030 Key fragment: 446 Elution shift as compared to 1030 caused by core α1,6-fucose
a3 a3	1151.45	A/F 7.2	A/F 7.2	A/F 9.5	[Fig 6H, sensitive to α1,2/3 mannosidase] Fits order of Golgi mannosidase II: Rose (2012) Curr Opin Struc Biol 22, 558 Elution: Tomiya, et al. (1991); 7.49 g.u. Key fragment: 827
	1192.47 (MGn)	A/F 7.0	A/F 6.8	A/F 8.4	[Fig 3J, resistant to α1,2/3 mannosidase] [Fig 7B, sensitive to FDL] For elution see also: Gutternigg, et al. (2007) J Biol Chem 282, 27825
	1192.47 (GnM)	A/F 10.3	A/F 9.0	F/A 16.0	[Fig 3F and Fig 7D, sensitive to α1,2/3 mannosidase] Resistant to FDL For elution see: Gutternigg, et al. (2007)
PC—	1195.47	A/F 7.7	A/F 5.5	A/F 6.9	Based on structure of m/z 1030 Due to PC, C18 elution later and Kinetex/amide elution earlier Key fragments: 369; 531; HF sensitive
a6 a3 a3	1265.51	A 8.0	A 6.8	A 9.2	Only found in PNGase A released pool. Key fragments: 446 and 592 [Fig 4H] [Fig 6H and I, sensitive to $\alpha 1,2/3$ mannosidase and HF (loss of 2 fucose residues)]
a3   a3	1265.51	A 9.8	A 8.0	A 11.2	Only found in PNGase A released pool. Key fragments: 446; 592 [Fig 6J] [Fig 6K, sensitive to HF, loss of 308 and 146]
α <sub>α</sub> α <sub>β</sub>	1281.51 (MMF <sup>3</sup> F <sup>6</sup> )	A 7.2	A 6.8	A 8.5	Only found in PNGase A released pool. [Fig 5B and C, sensitive to bovine fucosidase and HF, loss of 1 fucose residue] Key fragments: 446 and 592
a3 a3	1297.50	A 5.0	A 5.0	A 5.8	Only found in PNGase A released pool. Resistant to α1,6 mannosidase Based on structure of Man4GlcNAc2 Key fragments: 446

αδ	1297.50	n.d.	F 11.0	A/F >20.0	Key fragment: 608 [Fig 4G] Late elution also typical of 'GalFuc' (11.3 g.u.; Takahashi <i>et al.</i> (2003) <i>Eur J Biochem 270, 2627</i> ) Sensitive to <i>Aspergillus</i> β-galactosidase [Suppl. Fig 2I]
	1313.50 (Man5)	A/F 7.0	A/F 7.2	A/F 8.7	[Fig 3J, sensitive to α1,2/3 mannosidase, loss of 1 mannose residue] Tomiya, et al. (1991); 7.25 g.u. Key fragment: 827 Consistent with class I mannosidase processing of Man9
iz6	1338.53 (MGnF <sup>6</sup> )	A/F 10.3	A/F 8.6	A/F 13.0	[Fig 3F and Fig 7D, resistant to α1,2/3 mannosidase] Tomiya <i>et al.</i> (1988) <i>Anal Biochem 171</i> , 73; 10.2 g.u. Sensitive to FDL; Key fragment: 446
α6	1338.53 (GnMF <sup>6</sup> )	F 17.0	F 12.0	A/F >25.0	[Fig 3H, sensitive to α1,2/3 mannosidase] Tomiya <i>et al.</i> (1988); 12.7 g.u. Key fragment: 446
PC————————————————————————————————————	1341.53	F 13.0	A/F 7.7	A/F 11.5	Based on m/z 1030 and 1176 Key fragments: 369; 531 Elution shift as compared to 1195 caused by core α1,6-fucose.
PC—	1357.53	A/F 8.0	A/F 6.2	A/F 7.5	Key fragments: 369, 531[Fig 8A] [Fig 9B and C, sensitive to HF and FDL, sequential loss of PC and GlcNAc]
PC—	1357.53	A/F 11.5	A/F 8.7	A/F 14.5	[Fig 6D and F, sensitive to α1,2/3 mannosidase and HF] Key fragments: 369; 531
	1395.55 (GnGn)	A/F 9.0	A/F 8.0	A/F 11.5	Based on retention time on RP-HPLC and MS/MS Key fragments: 365; 1030 Sensitive to FDL For elution see: Gutternigg, et al. (2007)
	1427.56	A 11.5	A 8.2	A 12.7	Only found in PNGase A released pool. Key fragments: 446; 608; 754 [Fig 4I] [Fig 6B β-galactosidase sensitive; Fig 6D and E, resistant to α1,2/3 mannosidase and bovine fucosidase; Fig 6F, HF sensitive, loss of 146 (Fuc) and 308 (Hex+Fuc)]
α3	1459.56	A 4.7	A 5.0	A 5.4	Only found in PNGase A released pool. Based on m/z 1313 Key fragments: 446; 973
	1475.55	A/F 5.5	A/F 6.1	A/F 6.5	Sensitive to $\alpha 1,2/3$ mannosidase Key fragment: 827 Tomiya, et al. (1991); 6.18 g.u. (as compared to isomer with $\alpha 1,2$ -Man on middle mannose which elutes at 7.82 g.u.)
	1475.55	A/F 5.9	A/F 6.3	A/F 6.9	Key fragment: 989 [Fig 3B, sensitive to α1,2/3 mannosidase, loss of two mannose residues] Tomiya, et al. (1991); 6.15 g.u. Consistent with class I mannosidase processing of Man9
α6	1484.59	A 9.0	A 7.8	A 11.2	Only found in PNGase A released pool. Key fragments: 446, 592 HF sensitive, loss of 146 Sensitive to $\alpha$ 1,2/3 mannosidase
PC PC	1503.59	A/F 13.0	A/F 8.0	A/F 11.5	Key fragments: 369; 531 [Fig 8B] [Fig 9E and F, sensitive to HF and FDL, sequential loss of PC and GlcNAc] No loss of Fuc with HF; α1,6-Fuc shown with strong 446 fragment after HF

PC————————————————————————————————————	1503.59	A/F 19.0	A/F 11.0	F >25.0	Key fragments: 369; 531 Later elution as compared to other 1503 isomer due to modification of α1,6-
					mannose
	1541.61 (GnGnF <sup>6</sup> )	F 14.0	F 10.5	F 21.0	Order of elution (between the two 1338 isomers; 12.3 g.u; Tomiya <i>et al.</i> (1988)) Key fragments: 365; 446; 1030
PC	1560.61	F 8.0	F 6.8	F 8.8	Key fragments: 369; 572 [Fig 8C] Resistant to α1,6 mannosidase and FDL
PC—	1560.61	F 10.3	F 7.5	F 9.5	Key fragments: 369; 531 [Fig 8D] Resistant to FDL, sensitive to HF [Fig 7C, resistant to α1,2/3 mannosidase]
	1598.63	A/F 6.5	F 6.3	F 7.5	Key fragments: 569; 1030 [Fig 7E] Sensitive to FDL [Fig 7B] Biosynthesis: homologue of GnT V present in nematode N-glycome
	1637.60	A/F 5.0	A/F 5.5	A/F 5.3	Key fragment: 989 Tomiya, <i>et al.</i> (1991); 5.08 g.u. (as compared to isomer with α1,2-Man on middle mannose eluting at 6.72 g.u.)
	1637.60	A/F 5.7	A/F 6.1	A/F 6.2	Key fragment: 1151 Tomiya, et al. (1991); 5.83 g.u. Biosynthesis: both Man7 isomers consistent with prior action of ER mannosidase
PC a6	1649.64	A/F 9.8	A 7.2	A 10.0	Only found in PNGase A released pool. Key fragments: 369, 531 [Fig 8G] Bovine fucosidase and HF sensitive [Fig 9J and K; loss of PC and Fuc] Biosynthesis: transfer of fucose by FUT-1 to GnM and not MGn (Paschinger, et al. (2004) J Biol Chem 279, 49588)
PC PC	1706.67	F 17.0	F 9.5	F 16.0	Key fragments: 369; 531 Core α1,6-Fuc as only present in PNGase F released pool; later elution as compared to 1560.
PC————————————————————————————————————	1725.66	A/F 15.0	A/F 6.8	A/F 9.5	Sensitive to HF (loss of two PC) Key fragments: 369; 531 [Suppl. Fig. 5]
a6	1744.69	n.d.	F 8.2	F 11.5	Key fragments: 446; 1176 Based on 1598; later elution caused by core α1,6-fucose
PC-	1763.69	F 7.2	F 5.5	F 6.3	Key fragments: 369; 734 [Fig 8E] [Suppl. Fig. 4A]
	1763.69	n.d.	F 5.9	F 6.9	Key fragments: 369; 531; (no 734); 1195
PC————	1799.66 (Man8B)	A/F 4.6	A/F 5.2	A/F 5.0	Key fragment: 1151 Tomiya, et al. (1991); 4.76 g.u. [Suppl. Fig. 1] Biosynthesis: Man8B isomer consistent with prior action of ER mannosidase

	1799.66 (Man8A)	A/F 5.3	F 5.8	A/F 6.0	Key fragment: 989 Tomiya, <i>et al.</i> (1991); 5.65 g.u. [Suppl. Fig. 1]
PC PC	1871.72	A/F >20	F 8.6	F 14.5	Key fragments: 369; 531 Based on 1725; later elution caused by core α1,6-fucose.
PC- according to the second se	1909.75	n.d.	F 6.8	F 8.8	Key fragments: 369; 531; 734 [Suppl. Fig. 4B] Elution shift as compared to 1763 caused by core α1,6-fucose
PC————————————————————————————————————	1928.74	F 17.0	F 4.0	F 5.0	Key fragments: 369; 899 [Suppl. Fig. 4C]
PC-	1928.74	n.d.	n.d.	F 5.7	Key fragments: 369; 531; 734 [Suppl. Fig. 4D]
	1961.71 (Man9)	A/F 4.9	A/F 5.5	A/F 5.3	Tomiya, et al. (1991); 5.33 g.u. Key fragment: 1151 Biosynthesis: typical Man9 resulting after action of glucosidases I and II on typical transferred Glc <sub>3</sub> Man <sub>9</sub> GlcNAc <sub>2</sub>
PC————————————————————————————————————	2074.80	F > 20.0	n.d.	F 6.9	Key fragments: 369; 899 [Suppl. Fig. 4E] Elution shift as compared to 1928 caused by core α1,6-fucose
PC-	2074.80	n.d.	F 6.3	F 8.0	Key fragments: 369; 734 [Suppl. Fig. 4F] Elution shift as compared to 1928 caused by core $\alpha$ 1,6-fucose
PC—PC—PC—PC—PC—PC—PC—PC—PC—PC—PC—PC—PC—P	2093.80	A/F >20.0	A/F 4.0	F 4.8	Key fragments: 369, 899 [Fig 8F] Sensitive to HF, loss of three PC residues [Fig 9H]
	2123.76	F 6.0	F 6.6	F 6.7	Biosynthetic pathways Key fragment: 1313 Tomiya, et al. (1991); 6.29 g.u. Shift to later retention time as compared to Man9 observed for proven glycan from Dictyostelium M31 mutant (Hykollari et al. (2014) Electrophoresis 35, 2116
PC—  PC—  PC—  PC—  PC—  PC—  PC—  PC—	2239.86	F >20.0	F 4.5	F 6.7	Key fragments: 369; 899 Sensitive to HF, loss of three PC residues; after HF, fragmentation as for 1744 with strong 446 fragment indicative of core α1,6-Fuc [Suppl.Fig 6]
	2285.81		F 6.3	F 6.6	Biosynthetic pathways (trace amount) Key fragment: 1475 Shift to later retention time as compared to Man9 observed for proven glycan from Dictyostelium M31 mutant (Hykollari et al. (2014) Electrophoresis 35, 2116
	2447.86		F 6.5	F 6.9	Biosynthetic pathways (trace amount) Key fragment: 1637