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N-glycan structures of murine hippocampus serine protease, neuropsin, produced in *Trichoplusia ni* cells

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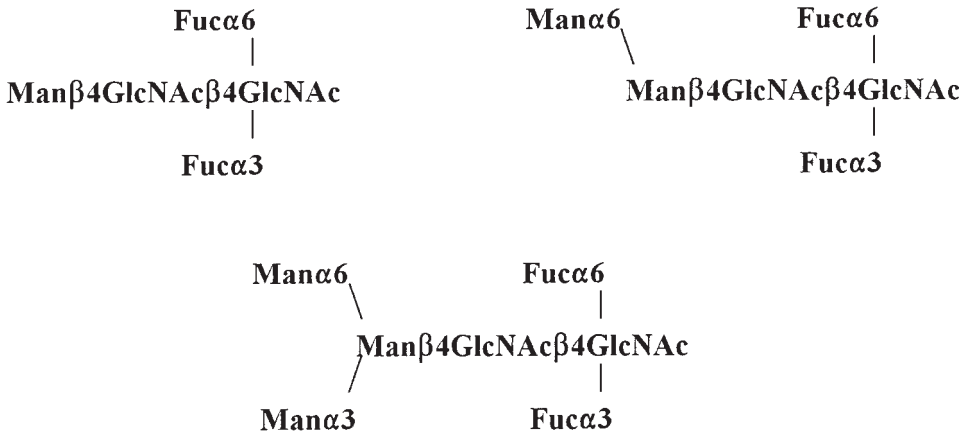
N-glycans of neuropsin (serine protease in the murine hippocampus) expressed in *Trichoplusia ni* cells were released from the glycopeptides by digestion with glycoamidase A (from sweet almond), and the reducing ends of the oligosaccharides were reductively aminated with 2-aminopyridine. The derivatized N-glycans were separated and structurally identified by a two dimensional high-performance liquid chromatography (HPLC) mapping technique on two kinds of HPLC columns. Fourteen different major N-glycan structures were identified, of which 6 were high-mannose type (9.1%), and the remaining 8 were paucimannosidic type. The presence of insect specific N-glycan structures containing both α1,3- and α1,6- di-fucosylated innermost N-acetylglucosamine residue (23.3%), as below, was also confirmed by 600 MHz ^{1}H-NMR spectroscopy.



Keywords: **neuropsin, N-glycan structures, insect cells, 2/3-D sugar mapping**

Abbreviations: **Fuc, L-fucose; HPLC, high performance liquid chromatography; Glc unit, glucose unit; PA, 2-pyridylamino-; ODS, octadecylsilica; *T. ni, Trichoplusia ni;* 2-D, two dimensional**

Introduction

Insect cells have been widely utilised as hosts for the production of various glycoproteins through the baculovirus expression system [1]. However, at this point it is not clear whether the N-glycan structures produced by insect cells are identical with the original N-glycan structures or not. If the structures are different, the conformation of the glycoprotein might also be very different and thus, the function of the insect glycoprotein should be different as well. This may have dramatic consequences for the applicability of the recombinant structures. However, general information on N-glycan structures and biosynthetic pathways in

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insects is still limited. A first and most urgent step is to accumulate structural data of N-glycans (both native and recombinant) in insect cells, as much as we can. Any discussion of eventual future practical, especially medical applications before having enough such data may lead to misleading conclusions. The evidence for the existence of characteristic difucosyl trimannosyl core structures in N-glycans is accumulating in both natural and recombinant insect cells [2–4], and in a nematode [5]. The purpose of this paper is to provide such data from a glycoprotein: neuropsin (M_r25032).

Neuropsin found in the mouse brain is a novel serine protease. It is a typical glycoprotein with Asn-95 as its only glycosylation site. Both brain neuropsin and recombinant neuropsin produced in the baculovirus/insect cell system have been reported to have significant limbic effects by changing the extracellular matrix environment [6,7]. In this paper we tried to elucidate the detailed structures of neuropsin N-glycans synthesized in insect cells. This would permit future comparison with the N-glycan structures of original mouse brain neuropsin (not elucidated yet).

Materials and methods

Materials

Glycoamidase A (glycopeptidase A) from sweet almond [8] was from Seikagaku Kogyo (Tokyo, Japan). Trypsin, chymotrypsin were from Sigma Chemical Co. (St. Louis, MO). α-L-Fucosidase from bovine kidney was purchased from Boehringer Mannheim (Mannheim, Germany), α-mannosidase from jack bean and the pyridylamino (PA) derivatives of isomalto-oligosaccharides from 4 to 20 (degree of polymerization of glucose residues) and of reference oligosaccharides (Code Nos. 000.1, 010.1, 100.2, 110.1, M5.1, M6.1, M7.1, M7.2, M8.1, and M.9.1, see the structures in Table 1) were from Seikagaku Kogyo, 010.0 was obtained by partial α-mannosidase digestion of 010.1.

Preparation of recombinant neuropsin glycoprotein

Neuropsin was expressed in baculovirus-infected High Five insect cells (BaculoGold Transfection Kit, PharMingen, San Diego, CA) and purified by two-column chromatographic steps using S-sepharose and heparin sepharose as described [9,10].

Preparation, derivatization, and characterization of N-glycans from insect neuropsin

Purified neuropsin (5 mg of protein correspond to 200 nmol of oligosaccharidales) was heated at 100 °C for 10 min. After digestion of neuropsin glycoprotein with trypsin and chymotrypsin (each 1%, w/w, of the substrate protein), at pH 8.0, the peptide/glycopeptide mixture was treated with glycoamidase A (0.6 mU) in 30 µl of 0.5 M citrate/phosphate buffer at pH 4.0 for 16 h to release the N-glycans. The mixture was finally digested with 1% (w/w) of pronase to convert peptide materials to amino acids and small peptides. Since no acidic residue was detected in the N-glycan mixture on the preliminary experiment, the N-glycan fraction was passed successively through a column of Dowex-50WX8 (H^+1, 1 ml) and a column of Dowex-1 (CO_3^{2-}, 1 ml) and throughly dried [11]. After reductive amination with 2-aminopyridine using sodium cyanoborohydride [12], the resultant pyridylamino (PA)-glycans were purified by gel filtration on a Sephadex G-15 column (1.0 × 40 cm). A 1/200 portion from each PA-glycan mixture was separated and characterized by high performance liquid chromatography (HPLC) using standard two-dimensional (2-D) sugar mapping technique [13,14], except that the amide column was used first, followed by the ODS column. In both columns, eluted PA-oligosaccharides were monitored by fluorescence (excitation, 320 nm; emission, 400 nm). The elution positions of the PA-oligosaccharides were expressed as glucose units (Glc unit), in reference to the PA-derivatized isomalto-oligosaccharides of polymerization degree from 4 to 20, and analysed by the 2-D mapping technique.

Exoglycosidase digestion procedure

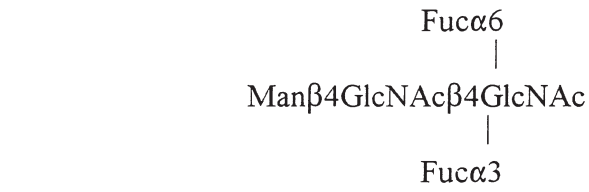
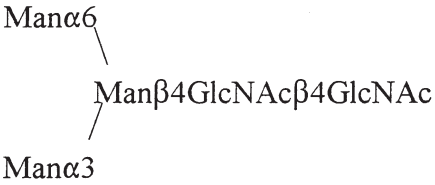
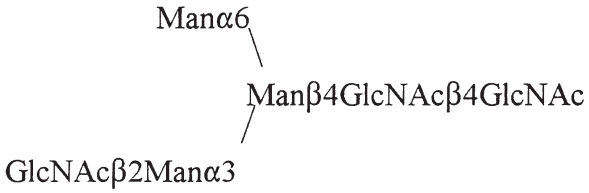
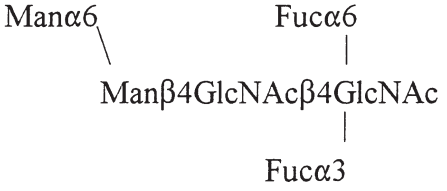
α-Mannosidase: The reaction mixture (final 20 µl) contained purified PA-glycan (50–500 pmol), α-mannosidase from jack bean (50 mU), 0.1M acetate buffer (pH 5.0) and 10 mM $ZnCl_2$. After incubation for 16 h at 37 °C, the reaction products were analysed by the 2-D mapping technique.

α-L-Fucosidase: The reaction mixture (final 20 µl) contained purified PA-glycan (50–500 pmol), α-L-fucosidase from bovine kidney (20 mU) and 0.2M acetate buffer (pH 4.5). After incubation for 2–20 h at 37 °C, the reaction products were analysed by the 2-D mapping technique. After complete releasing of α-1,6 linked fucose residue, α-1,3 linked fucose residue was released. Therefore, from both possible N-glycans (the one containing only α-1,6 linked fucose and the one containing only α-1,3 linked fucose), only the latter was present as an intermediate in the reaction mixture.

^{1}H NMR measurements

PA-oligosaccharides **glycans C-2** and **E** (6 nmol each) isolated by HPLC were desalted by gel filtration on a Sephadex G-15 column. Each sample was dissolved in 99.996 % D_2O (Cambridge Isotope Laboratories, Andover, MA). 600 MHz ^{1}H-NMR spectra were recorded using a Bruker DRX-600 spectrometer at a probe tem-

Table 1. Chromatographic properties and the proposed structures of PA-*N*-glycans obtained from neuropsin expressed in insect cells

Glycan name *Code no.*	*PA-N-glycans*	*Glc units* *ODS* *Amide*	*Relative quantity* *(mol %)*
A-1 M1.1FF		7.7 3.5	2.8
A-2 010.0		10.1 3.5	40.4
B 000.1		7.4 4.2	2.1
C-1 100.2		7.4 4.6	2.0
C-2 M2.1FF		8.7 4.6	9.9

(continued)

perature of 60 °C. Chemical shifts are given relative to external acetone (2.213 ppm) [15]. 1D-HOHAHA spectra [16] were recorded by selectively exciting the H-1 resonances of Man residues using a rectangular pulse of 70 ms duration. The mixing time was 100–200 ms at a field strength of 8.3 kHz. The trim pulse of 2.5 ms duration was applied at the beginning and end of the MLEV-17 spin-lock period.

Results and discussion

PA-N-glycan profile of neuropsin glycoprotein and the structural characterization using 2-D mapping technique

N-glycans of recombinant neuropsin released from the glycoprotein with glycoamidase A were derivatized with 2-aminopyridine. Then we first applied the PA-glycan

Table 1. *(continued)*

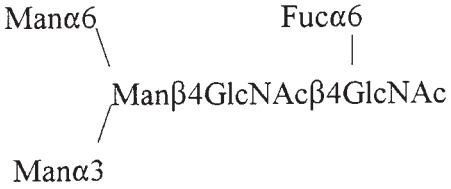
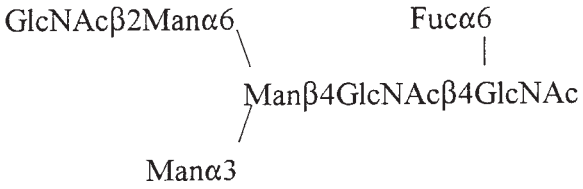
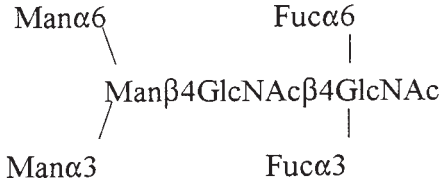
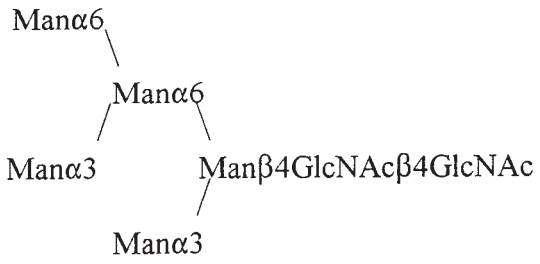
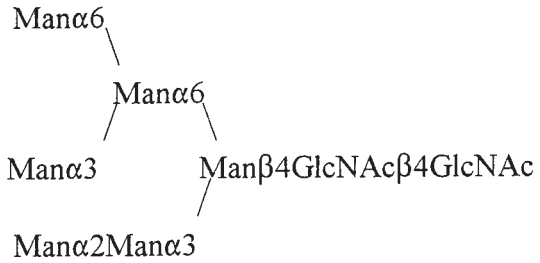
Glycan name *Code no.*	*PA-N-glycans*	*Glc units* *ODS* *Amide*	*Relative quantity* *(mol %)*
C-3 010.1		10.0 4.6	21.2
D 110.1		12.5 5.0	1.9
E 010.1F		8.6 5.5	10.6
F M5.1		7.1 6.1	4.2
G M6.1		6.0 7.0	2.6

Table 1. *(continued)*

Glycan name *Code no.*	*PA-N-glycans*	*Glc units* *ODS* *Amide*	*Relative quantity* *(mol %)*
H-1 M7.2	Manα2Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα3	5.1 8.1	0.4
H-2 M7.1	Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα2Manα3	5.8 8.0	0.4
I M8.1	Manα2Manα6 Manα6 Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα2Manα3	4.9 9.0	0.7
J M9.1	Manα2Manα6 Manα6 Manα2Manα3 Manβ4GlcNAcβ4GlcNAc Manα2Manα2Manα3	5.2 9.5	0.8

mixture on an amide column (Figure 1). Each fraction from A to J was further chromatographed on an ODS column. Fraction A (Glc unit 3.5 on the amide column) was separated on the ODS column into two **glycans A-1** and **A-2** (Glc unit 7.7 and 10.1, respectively). Similarly, fraction C (Glc unit 4.6 on the amide column) and fraction H (Glc unit 8.1 on the amide column) were separated on the ODS column into three **glycans C-1, C-2** and **C-3** (Glc unit 7.4, 8.7, and 10.0), and two **glycans H-1** and **H-2** (Glc unit 5.1 and 5.8), respectively. The other fractions were found to be homogeneous (data not shown). The two-dimensional mapping analysis showed that all oligosaccharides except for **A-1, C-2** and **E** coincided (± 5%) with one of the known oligosaccharides. Thus **glycans A-2, B, C-1, C-3, D,**

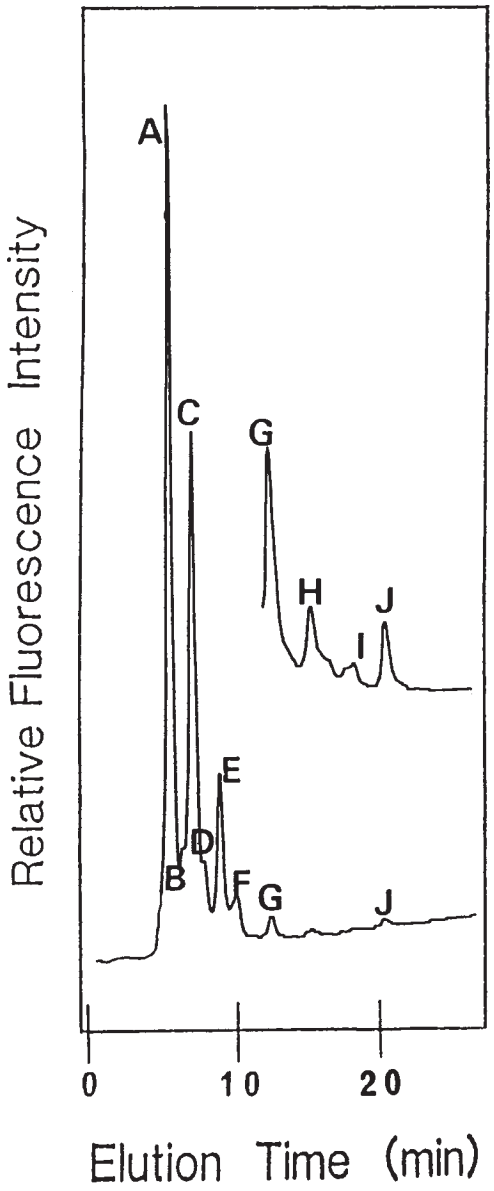
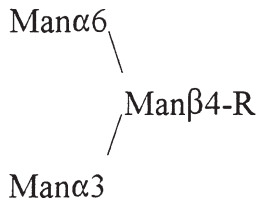


Figure 1. HPLC profile of PA-oligosaccharides derived from neuropsin. The oligosaccharides were released with glycoamidase A from neuropsin expressed in insect cells and were pyridylaminated and subjected to separation on an amide-silica column.

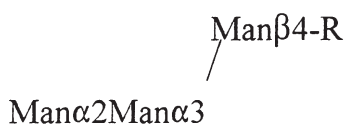
F, G, H-1, H-2, I, and **J** were assigned as code nos. 010.0, 000.1, 100.2, 010.1, 110.1, M5.1, M6.1, M7.1, M7.2, M8.1 and M9.1, respectively (Table 1). Co-chromatography (on the ODS- and amide-columns) for each of the sample PA-oligosaccharides **A-2, B, C-1, C-3, D, F, G, H-1, H-2, I,** and **J** with its corresponding reference PA-oligosaccharide confirmed the above assignment.

Identification of **glycans A-1**, **C-2**, and **E** (Figure 2)

Glycans A-1, C-2 and **E** were each eluted on a 2-D map at (7.7 on the ODS column and 3.5 on the amide column), (8.7, 4.6), and (8.6, 5.5), respectively (Figure 2). These coordinates did not coincide with any reference coordinates on the map. After α-mannosidase (jack bean) digestion, **glycan E** yielded two glycans and the resulting coordinates matched those of **glycans C-2** and **A-1.** The decreases of Glc units on the amide column from **glycan E** (5.5) to **C-2** (4.6) and further to **A-1** (3.5) were each about 1.0, corresponding to a loss of one mannose residue. This result suggests that the structural differences among **glycans E, C-2,** and **A-1** depend on the number of mannose residues. Although α-L-fucosidase (bovine kidney) preferentially removes the fucose residue α-1,6 linked to the PA-modified GlcNAc, it finally can also remove the other fucose residue α-1,3 linked to the same PA-GlcNAc. After the usual incubation time with α-L-fucosidase digestion of each **glycan E, C-2,** and **A-1,** we confirmed that the resulting coordinates of de-α-1,6-fucosyl oligosaccharides coincided with those of reference oligosaccharides code Nos. 000.1F (5.8 on the ODS column and 5.1 on the amide column), M2.1F (6.1, 4.2), and M1.1F (5.3, 3.0), respectively. These results were further confirmed by co-chromatography procedures. Subsequently, using a longer incubation time with the α-L-fucosidase, removal of the fucose residue α-1,3-linked to the PA-modified GlcNAc produced non-fucosyl oligosaccharides coeluted with code Nos. 000.1 (the same as **glycan B,** 7.4 on the ODS column and 4.3 on the amide column), M.2.1 (7.4, 3.3), and M1.1 (6.6, 2.0), respectively. Based on the known N-glycan structures, there can be four possible structures for the arrangement of the three mannosyl residues: (R is GlcNAcβ4GlcNAc)



Code No. 000.1 (Glc unit 7.4, 4.3)



Code No. M3.1(Glc unit 5.5, 3.9)

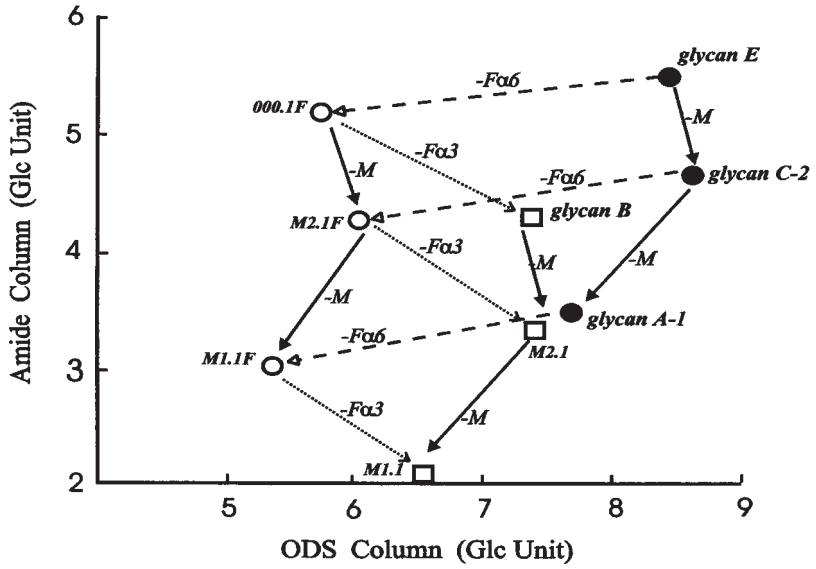
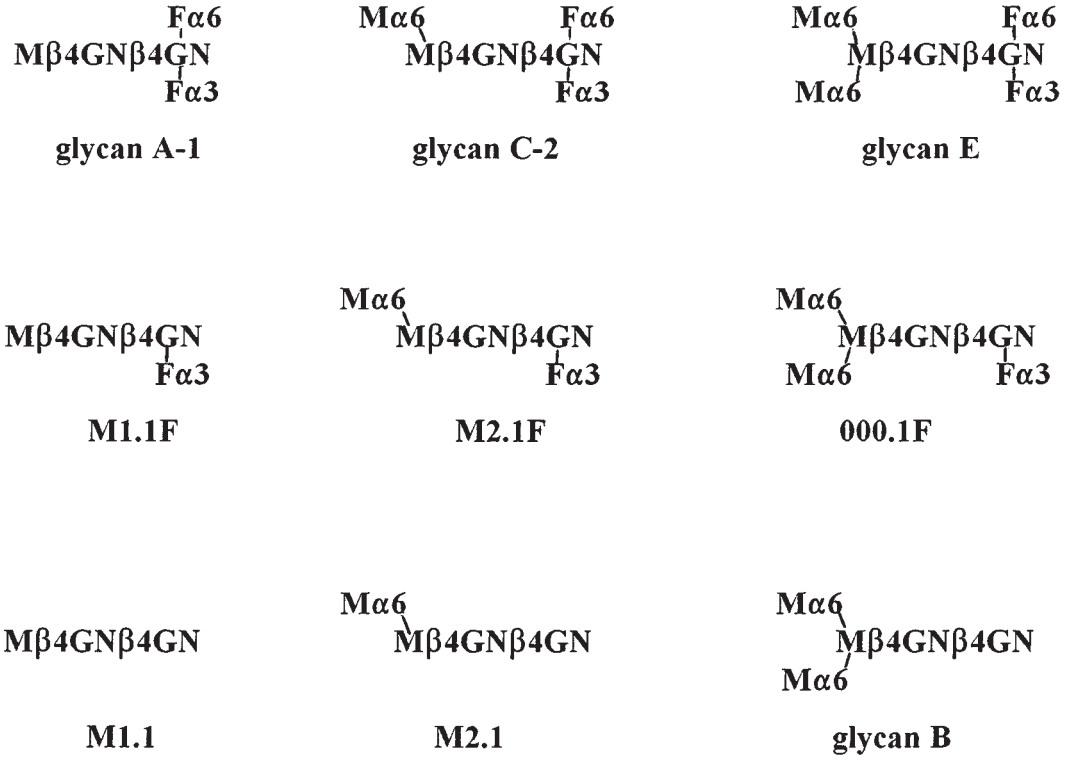
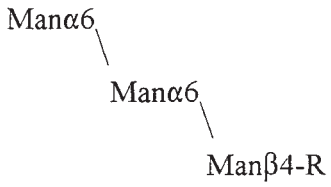
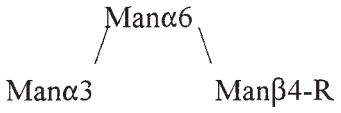


Figure 2. Structural characterization of **glycans A-1, C-2,** and **E** on the 2-D map. The arrows indicate the direction of changes in the coordinates of oligosaccharides after digestion with α-mannosidase (→), and α-fucosidase (α-1,6, ---▷; α-1,3, ···≫). ●, sample glycans (with both α-1,6 and α-1,3 fucose); ❍, reference glycans (with α-1,3 fucose only); and □, reference glycans (without fucose residue). Structures of the N-glycans derived from **glycans A-1, C-2** and **E** are shown below.



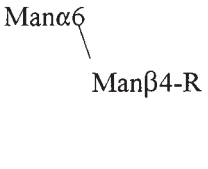


Code No. M3.2 (Glc unit 7.2, 4.3)

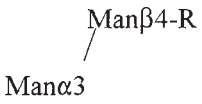


Code No. M3.3 (Glc unit 7.5, 4.1)

Similarly, there can be two possible structures for the arrangement of the two mannosyl residues:



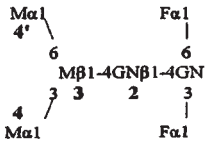
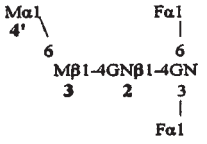
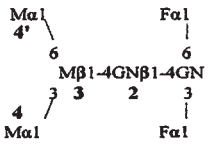
Code No. M.2.1(Glc unit 7.4, 3.3)



Code No. M2.2 (Glc unit 6.6, 3.0)

These four structures (for the three mannose containing N-glycans) or two structures (for the two mannose containing N-glycans) are discernible on the 2-D map [17,18]. Fig. 2 shows that on the 2-D map, the changes from **glycan E** to **A-1,** via **C-2,** from 000.1F to M1.1F, by way of M2.1F, and from **glycan B** to M.1.1, via M2.1, are parallel. These results suggest that our estimated structures are reasonable (Table 1).

Table 2. Chemical shifts of structural-reporter-group protons for pyridylamino derivatives of **glycans E** and **C-2** from neuropsin expressed in insect cells.

Reporter group	Residue	Chemical shift[a] in glycan E	glycan C-2	II-B[c]
				
H-1	GlcNAc-2	4.682	4.678	4.676
	Man-3	4.738	4.719	4.748[d]
	Man-4′	4.907	4.902	4.909
	Man-4	5.113	—	5.099
	Fucα1,3	5.051	5.049	5.121
	Fucα1,6	4.862	4.863	4.926
H-2	Man-3	4.219[b]	4.057[b]	4.257
	Man-4′	3.967[b]	3.959[b]	3.970[e]
	Man-4	4.062[b]	—	4.064

[a]Chemical shifts are given in ppm from external acetone (2.213 ppm) in D_2O at 60 °C.
[b]Obtained from a 1D-HOHAHA experiment at 60 °C.
[c]Oligosaccharide II-B was obtained from honeybee venom phospholipase A2 (2) recorded at 27 °C.
[d]Recorded at 42 °C.
[e]Obtained from a 2D-HOHAHA experiment at 42 °C.

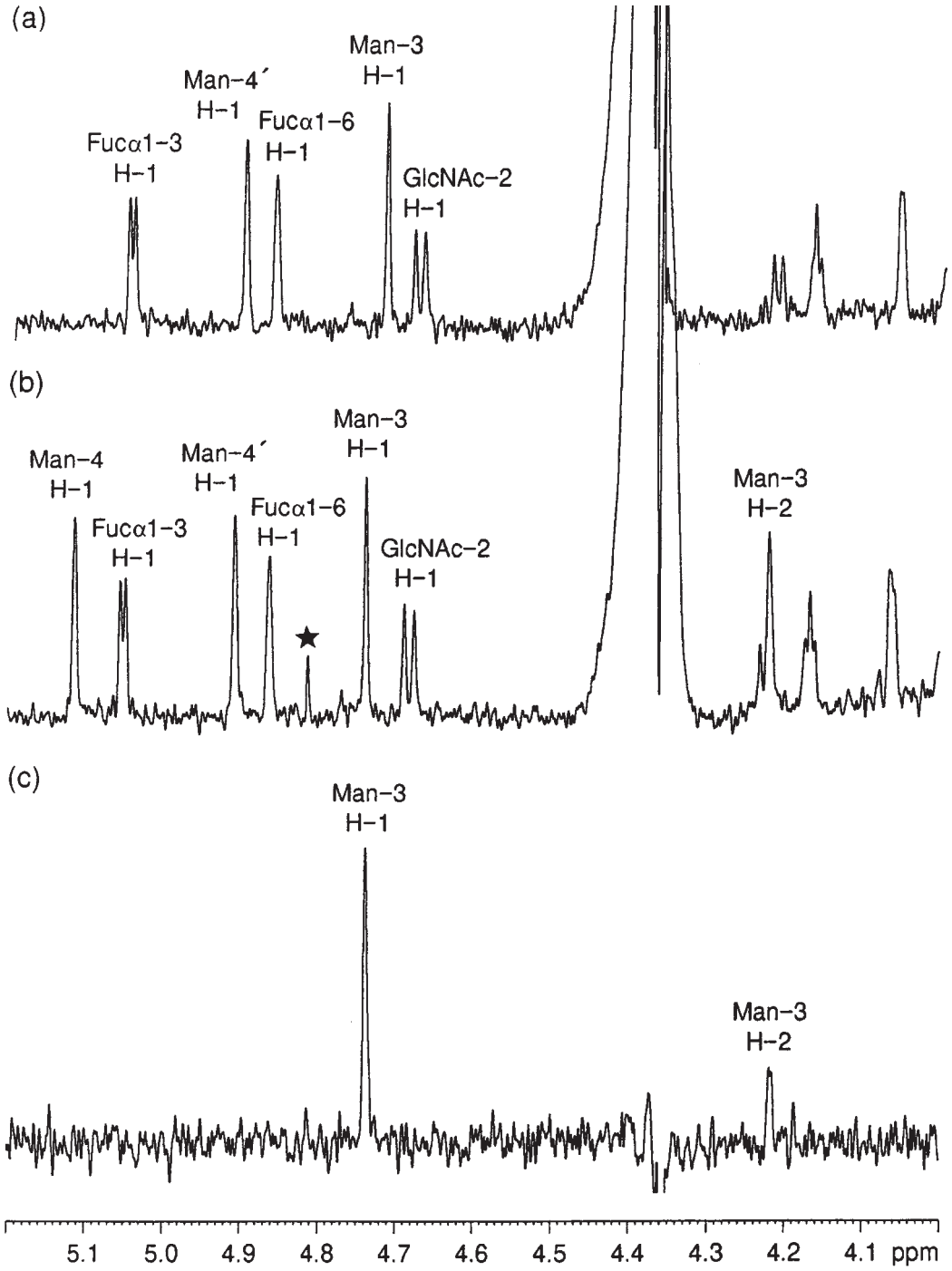


Figure 3. 600 MHz ^{1}HNMR spectra of **glycans C-2** and **E.** Regular spectrum of (a) **glycan C-2** and (b) **glycan E.** (C)1D-HOHAHA spectrum of **glycan E** recorded by selectively exciting the H-1 resonance of Man-3. The peak marked with an asterisk is due to the contaminant, judging from the fact that the intensity is less than one proton.

Structural determination with ^{1}H-NMR spectroscopy

These difucosyl *N*-glycan structures were eventually confirmed with ^{1}H-NMR spectroscopy. The chemical shift values for the structural reporter groups of **glycans E** and **C-2** are summarized in Table 2 and Figure 3. In Figures 3a and b, the anomeric regions of 600 MHz ^{1}H NMR spectra of **C-2** and **E** suggest that Fuc α-1,3 and α-1,6 residues,

GlcNAc-2, Man-3, and Man-4′ residues are all in the same environments, except that one additional Man H-1 resonance is observed at 5.113 ppm for **glycan E.** It is rather difficult to make an unambiguous assignment for this Man resonance solely on the basis of its H-1 chemical shift data, because either Man-4 or Man-A could give an H-1 resonance at this position. For identification of the linkage of the sugar residues, NMR information on through-bond correlation via long-range coupling or through-space correlation obtained from nuclear Overhauser effect experiments is known to be helpful. However, NMR spectra, which give us the information, could not be measured with sufficient S/N, due to a limited amount of the samples. Fortunately, it is possible to judge whether Man-4 exists, on the basis of the chemical shift of the Man-3 H-2 resonance: the H-2 resonance from Man-3 with and without Manα 1,3 branch have been observed at ca. 4.2 and at ca. 4.1 ppm, respectively [19]. In order to establish scalar connectivities between the H-1 and H-2 resonances for the individual Man residues, we used 1D-HOHAHA technique, which is able to apply even for a small amount of samples. The 1D-HOHAHA spectrum recorded by selectively exciting the Man-3 H-1 resonance clearly showed that the chemical shift value of the Man-3 H-2 was 4.219 ppm, indicating that Man-4 is contained in the **glycan E.** Thus, we assigned the resonance at 5.113 ppm to Man-4. In a similar way, connectivities between H-1 and H-2 resonances were established for all of the Man resonances originating the **glycans E** and **C-2.** In Table 2, a reference compound oligosaccharide II-B in honeybee venom phospholipase A2 is also shown. It was first reported by Staudacher et al. (2). They found it to be a naturally occurring N-glycan with two fucose residues linked to the same Asn-bound *N*-acetylglucosamine. In spite of the difference between the two experimental temperatures (27 °C and 60 °C), the chemical shifts all seem to have been essentially the same. The chemical shifts of the anomeric protons of **glycans E** and **C-2** clearly showed the existence of both α-1,3 and α-1,6 fucose residues at the same PA-*N*-acetylglucosamine residue, that is, the values of 5.051 and 4.862 ppm for **glycan E,** and 5.049 and 4.863 ppm for **C-2** were very close to those for reference compound II-B. Similarly, the existence of the H-1 and H-2 chemical shifts for Man-3 (4.738 and 4.219 ppm), for Man-4′ (4.907 and 3.967 ppm) and for Man-4 (5.113 and 4.062 ppm) in **glycan E** were essentially the same as those of II-B. These results combined with our above 2-D mapping findings confirm that the structures as summarized in Table 1 are reasonable. The structure of **glycan A-1** was not known before the present study to the best of our knowledge.

The elucidation of the effect of these N-glycan structures on protein conformation awaits further study.

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