Chemical characterization of milk oligosaccharides of the tiger quoll (*Dasyurus maculatus*), a marsupial

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Milk oligosaccharides were separated from the carbohydrate fraction of milk of the tiger quoll a species of marsupial that is closely related to the eastern quoll, *Dasyurus viverrinus*. They were characterized by ^{1}H – nuclear magnetic resonance spectroscopy and matrix – assisted laser desorption/ionization time-of-flight mass spectrometry. The following oligosaccharides were identified; Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)[Gal(β1-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novooctaose), Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)

Glc, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc with an α(2-3)Neu5Ac linked to β(1-4)Gal residue of either branch of Gal(β1-4)GlcNAc(β1-6) units, and Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc with a β(1-3) linked Gal and an α(2-3) linked Neu5Ac. In addition, larger oligosaccharides were characterized as follows; Gal(β1-3){Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)GlcNAc(β1-6)}Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc and Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3){Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)GlcNAc(β1-6)}Gal(β1-4)Glc and their α(2-3) linked Neu5Ac derivatives.

Keywords: Tiger quoll, Milk oligosaccharides, Marsupials

Introduction

Mammalian milk or colostrum contains from a trace to over 10% of carbohydrate in which the disaccharide lactose (Gal(β1-4)Glc) usually predominates over lower concentrations of a variety of oligosaccharides: these mostly have a lactose unit at their reducing ends [1,2]. In the milk of monotremes, marsupials and some Arctoidea species of Carnivora (ursids, mustelids, Pinnipeds), however, oligosaccharides usually predominate over free lactose [2-4]. Among marsupials, oligosaccharides have been characterized in the tammar wallaby (*Macropus eugenii*) [5-10], red kangaroo (*Macropus rufus*) [11], koala (*Phascolarctos cinereus*) [12], brushtail possum (*Trichosurus vulpecula*) [13], wombat (*Vombatus ursinus*) [14] and eastern quoll (*Dasyurus viverrinus*) [15]. The neutral oligosaccharides of milk of the tammar wallaby have been isolated and are characterized by the presence of a major linear series of galactosyllactoses ranging from Gal(β1-3)Gal(β1-4)Glc (3'-galactosyllactose) to Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc and a minor series of branched oligosaccharides containing β(1-6) linked GlcNAc including

Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) [5-9]. The acidic oligosaccharides of milk of the red kangaroo were found to contain non reducing N-acetylneuraminic acid or sulfate at OH-3 of non reducing Gal residues whose core structures were similar to the core structures of the neutral milk oligosaccharides of the tammar wallaby [11]. Most of the neutral and acidic milk oligosaccharides of the brushtail possum were found to be similar to the neutral oligosaccharides of the tammar wallaby and the acidic oligosaccharides of the red kangaroo, respectively [13]. In these milks, the linear oligosaccharides predominate over the branched oligosaccharides. The eastern quoll milk contains Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4) Glc (lacto-N-novooctaose) as well as 3'-galactosyllactose, Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, lacto-N-novopentaose I as neutral saccharides, and the N-acetylneuraminyl derivatives of lactose, lacto-N-novopentaose I and lacto-N-novooctaose with lacto-N-novopentaose I sulphate as acidic saccharides [15]. Thus in eastern quoll milk, branched oligosaccharides predominate over linear oligosaccharides.

In this study, we characterized the neutral and acidic milk oligosaccharides of the tiger quoll, which is closely related to the eastern quoll. It was of interest to compare the milk oligosaccharides of these two species of carnivorous marsupials.

Materials and methods

Milk carbohydrate sample and chemicals

The milk used in this study originated from an animal that had been caught in the wild in Tasmania and brought to the CSIRO Division of Wildlife Research, Canberra (ACT), as part of another project. Five milk samples, each about 100 µL, collected between September 25 and November 11, 1979, were pooled and the carbohydrate fraction extracted as previously described [16] and freeze-dried. The freeze-dried material was stored in sealed tubes at

-20℃ for about 35 years prior to analysis.

Separation of oligosaccharides by gel filtration and high performance liquid chromatography using a graphite carbon column.

The carbohydrate fraction (102 mg) of the tiger quoll milk was dissolved in 2 mL of water and the solution passed through a BioGel P-2 column (＜45 µm, 2.5×100 cm, Bio-Rad Laboratories, Hercules, CA) that had been calibrated with 2 mg of each galactose (monosaccharide), lactose (disaccharide), and raffinose (trisaccharide). The gel had been treated with 0.1 M HCl and 0.1 M NaOH before use. Elution was done with distilled water at a flow rate of 15 mL/h, and fractions of 5 mL were collected. Aliquots (0.5 mL) of each fraction were analyzed for hexose with phenol–H_2SO_4 [17] and for sialic acid with periodate – resorcinol [18]. Aliquots of fractions of the first eluted peak (see Fig. 1a, TQM-1 to TQM-3) were diluted with four volumes of distilled water and re-analyzed for hexose using monitored by phenol – H_2SO_4. Peak fractions were pooled as shown in Figs. 1a and 1b and freeze-dried. The saccharides in the peak fractions, TQM-1 to TQM-9 (see Figs. 1a and 1b) were checked by thin layer chromatography (TLC) using acetone/2-propanol/0.1 mol lactic acid (2:2:1, v/v) as a developing solvent. Detection of the spots was done by spraying with 5% H_2SO_4 in ethanol and heating. .

The components in TQM-6 and TQM-7 were characterized by ^{1}H-NMR spectroscopy. Those in TQM-8, TQM-5, TQM-4 and TQM-3 were first subjected to high performance liquid chromatography (HPLC) (chromatograms in Figs. 2a and 2b, and Figs. 3a and Fig. 3b, respectively), because these fractions were mixtures or contained contaminants. The Hitachi 7000 series HPLC system (Tokyo) consisted of an autosampler L-7200, a column oven L-7300, a pump L-7100, and an evaporation light scattering detector SEDEX-75 with a system controller D-7100. The HPLC stationary phase was a 7 µm Hypercarb carbon column (100 × 4.6 mm i.d.; Thermo Fisher Scientific, Waltham), while the mobile phase was acetonitrile

in distilled water run at 40℃. The LC gradient was delivered at 1.0 mL/min and consisted of an initial linear increase from 5 to 30% acetonitrile over 80 min. The oligosaccharides in each of the separated fractions were pooled, lyophilized and characterized by ^{1}H-NMR spectroscopy and MALDI-TOF mass spectrometry.

The components in peaks TQM-1 and TQM-2 of the gel chromatogram (Fig. 1b) that reacted positively with both periodate – resorcinol (630 nm) and phenol – H_2SO_4 (490 nm) were each dissolved in 2 mL of 50 mmol/L Tris hydroxyaminomethane – HCl buffer solution (pH 8.7) and subjected to anion exchange chromatography on a DEAE-Sephadex A-50 column (2.0 × 35 cm; GE Healthcare, Uppsala, Sweden) that was equilibrated and eluted with the same solution. Elution was done at a flow rate of 15 mL/h and fractions were analyzed for hexose using phenol – H_2SO_4. Fig. 4 shows that the ion exchange chromatography of TQM-1 had separated this fraction into two unadsorbed peaks designated TQM-1-1 and TQM-1-2 (Fig. 4b), while that of TQM-2 had only one eluted peak re-designated TQM-2 (Fig. 4a). The components in these were pooled, lyophilized, dissolved in 2 mL of water, and passed through a column (2.0 × 35 cm) of BioGel P-2 to remove salts, as described above. The components in TQM-1-1 were separated by HPLC using the Hypercarb carbon column. The HPLC was performed as described above, except that the LC gradient consisted of an initial linear increase from 15 to 40% acetonitrile over 80 min (chromatogram in Fig. 3c). The oligosaccharides in the separated fractions were pooled, lyophilized and characterized by ^{1}H-NMR spectroscopy and MALDI-TOF mass spectrometry.

Separation of the oligosaccharides by normal phase high performance liquid chromatography

The components in TQM-2 and TQM-1-2 fractions obtained by the ion exchange chromatography were subjected to HPLC on a TSK gel Amide-80 column (4.6 × 250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Japan (chromatogram in Fig. 5a and 5b). The mobile phase was 50% and 80% (vol/vol) acetonitrile in 15 mmol/L potassium phosphate buffer (pH 5.2).

Elution was done using a linear gradient of acetonitrile from 80 to 50% at 60℃ at a flow rate of 1 mL/min. The eluates were designated as peaks TQM-2-1 to TQM-2-4 and TQM-1-2-1 to TQM-1-2-15 (Fig. 5a and 5b) which were each pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy and MALDI-TOF mass spectrometry to determine their structures.

^{1}H-NMR spectroscopy

Nuclear magnetic resonance spectra were recorded in D_2O (99.96 atom D%; Aldrich, Milwaukee, WI) at 500 or 600 MHz for ^{1}H-NMR with a JEOL ECP-500 Fourier transform-NMR (Jeol, Tokyo, Japan) or a Varian INOVA 600 spectrometer)Varian Inc, Palo Alto, CA) operated at 293.1 K. Chemical shifts are expressed as change relative to internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt, but measured by reference to internal acetone (δ = 2.225).

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MA) was performed on the oligosaccharide fractions, using an Autoflex Ⅱ TOF/TOF mass spectrometer (Brucker Daltonics, Bremen, Germany). Lyophilized oligosaccharide fractions were dissolved in 5 μL of milli-Q water. The oligosaccharide solution was mixed with an equal volume of 10 mg/mL. SDHB)Brucker Daltonics), which is a mixture of 2,5-dihydrobenzoic acid and 2-hydroxy-5-methoxybenzoic acid, saturated in milli-Q water, spotted on a MTP 384 target plate ground steel TF (Bruker Daltonics), and dried. Mass spectra were obtained using a pre-installed method, RP__0-2 kDa (a reflector positive ion mode focusing on the mass range up to 2 kDa). Peptide calibration standard Ⅱ (Bruker Daltonics) was used for external calibration of the mass spectrometer.

Results

Separation and characterization of neutral saccharides

The crude carbohydrate fraction (total 102 mg) from tiger quoll milk separated into several peaks, designated as TQM-1 to TQM-9 during gel filtration on BioGel P-2 as shown in Figs. 1a and 1b. Since the components in TQM-3 to TQM-9 (Fig. 1a and 1b) did not react positively with periodate – resorcinol, they were considered to contain neutral oligosaccharides. During the ion exchange chromatography the components in TQM-1 separated into two fractions designated as TQM-1-1 and TQM-1-2 (Fig. 4b), while the components in TQM-2 eluted as one peak, re-designated TQM-2 (Fig. 4a). The components in TQM-8, TQM-5, TQM-4, TQM-3 and TQM-1-1 were subjected to HPLC using a Hypercarb carbon column and the resulting peaks designated as shown in Figs 2 and Fig. 3. The separated peak components obtained by gel filtration and HPLC were characterized by their ^{1}H-NMR and MALDI-TOF MS spectra. The characterizations were performed only for those for which clear ^{1}H-NMR and MALDI-TOF MS spectra were obtained, except for oligosaccharides whose ^{1}H-NMR spectra were similar to those previously found in other marsupial milks [12-15].

TQM-8-4 and TQM-8-5, TQM-7, TQM-6, TQM-5-1 and TQM-5-2, TQM-4-6 and TQM-4-8, TQM-4-4 and TQM-4-5, TQM-4-7, and TQM-3-1

From agreement of ^{1}H-NMR spectra (chemical shifts in Supplemental Tables 1 and 2) with those of authentic saccharides as well as the published data [12-15], the oligosaccharides in these fractions were characterized to be as follows; lactose (TQM-8-4 and TQM-8-5), 3'-galactosyllactose (TQM-7), 3",3'-digalactosyllactose (TQM-6), lacto-N-novopentaose I (TQM-5-1 and TQM-5-2), galactosyl lacto-N-novopentaose I (TQM-4-6 and TQM-4-8), galactosyl lacto-N-novopentaose II (TQM-4-4 and TQM-4-5), galactosyl lacto-N-novopentaose III (TQM-4-7), and lacto-N-novooctaose (TQM-3-1 and TQM-3-2) (Table 1).

TQM-2 and TQM-1-2

The oligosaccharides in fractions TQM-2 and TQM-1-2 were each separated by subjected to HPLC with an Amide-80 column, the results of which are shown in Fig. 5. Fraction TQM-2 yielded 4 peaks of which only TQM-2-3 was studied, while TQM-1-2 yielded 15 peaks, of which only TQM-1-2-1, TQM-1-2-2 and TQM-1-2-4 were studied.

TQM-2-3

The MALDI-TOF MS of the oligosaccharides in TQM-2-3 had the MS ion at 1962 of [M+K], showing the monosaccharide composition to be that of an undecasaccharide. $[Hex]_8[HexNAc]_3$. This monosaccharide composition suggested that these saccharides contained two Gal and one GlcNAc additional to lacto-N-novooctaose.

The ^{1}H-NMR spectrum (Fig. 6, chemical shifts in Supplemental Table 3) had the anomeric shifts of α-Glc, two internal β(1-3) linked Gal, β-Glc, two β(1-6) linked GlcNAc, two external β(1-3) linked Gal and β(1-6) linked GlcNAc at δ 5.228, 4.697 and 4.686, 4.674, 4.652 and 4.645, 4.618 and 4.614, and 4.592, respectively. The spectrum had the H-1 shifts of β(1-4) linked Gal, which were substituted by β(1-3) linked Gal, at δ 4.526 and 4.503, and the external β(1-4) linked Gal at δ 4.476 and 4.469. It had the H-4 of β(1-3) or β(1-4) linked Gal, which were substituted by β(1-3) linked Gal, at δ 4.138, 4.169 and 4.180 and it had the NAc shifts of β(1-6) linked GlcNAc at δ 2.073, 2.068, 2.065 and 2.045. From these observations it was concluded that the oligosaccharides in TQM-2-3 contained Gal(β1-3) and Gal(β1-4)GlcNAc(β1-6) additional to lacto-N-novooctaose.

The data suggested three possible structures for these oligosaccharides as shown in Fig. 7. By observing the shift intensities of NAc of β(1-6) linked GlcNAc in the inserted portion of the NMR (see Fig. 6) it was evident that the total intensity of the shifts at δ 2.073, 2.068 and 2.065 was twice as high as that at δ 2.045. This suggested that the former three shifts arose from NAc of β(1-6) linked GlcNAc of GlcNAc(β1-6)Gal(β1-4)Glc(NAc) unit, while

the latter arose from that of a GlcNAc(β1-6)Gal(β1-3)Gal unit. These observations led us to the tentative conclusion that the two structures enclosed by the frame in Fig. 7 were more plausible than the third structure.

TQM-1-2-1

The MALDI-TOF MS of the oligosaccharides in TQM-1-2-1 had the MS ions at 1726 and 1764 of [M+K] and [M+2K-H], respectively, indicating a monosaccharide composition of $[Neu5Ac]_1[Hex]_6[HexNAc]_2$.

As the ^{1}H-NMR spectrum (chemical shifts in Supplemental Table 3) was essentially similar to that of DV-1-2-10 which had been separated from eastern quoll milk, the oligosaccharides in this fraction were characterized to be the nonasaccharides Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc and Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc with an α(2-3)Neu5Ac linked to β(1-4) Gal residue of either branch of the two Gal(β1-4)GlcNAc(β1-6) units. However, the spectrum had additional H-1 shifts of two β(1-4) linked Gal at δ 4.506 and 4.467 compared with that of DV-1-2-10. In this study the spectrum was obtained at 600 MHz, while that of DV-1-2-10 was obtained at 500 MHz suggesting that the additional shifts were observed as a result of higher resolution of the spectrum at 600 MHz.

TQM-1-2-2

The MALDI-TOF MS of the oligosaccharides in TQM-1-2-2 had the MS ions at 1888 and 1926 of [M+K] and [M+2K-H], respectively, indicating a monosaccharide composition of $[Neu5Ac]_1[Hex]_7[HexNAc]_2$.

The ^{1}H-NMR spectrum (chemical shifts in Supplemental Table 3) had H-3 axial, H-3 equatorial and NAc shifts at δ 1.801, 2.760 and 2.029, respectively, indicating the presence of α(2-3) linked Neu5Ac. The spectrum had the anomeric shifts of α-Glc, internal β(1-3) linked Gal, β-Glc, β(1-6) linked GlcNAc, external β(1-3) linked Gal and β(1-6) linked GlcNAc at δ

5.228, 4.688, 4.672, 4.644, 4.618 and 4.596, respectively. It had the H-1 shift of ß(1-4) linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.554, the H-1 of ß(1-4) linked Gal, which was substituted by ß(1-3) linked Gal, at δ 4.522, and the H-1 of external ß(1-4) linked Gal at δ 4.488, 4.475 and 4.468. The spectrum had the H-4 of ß(1-3) or ß(1-4) linked Gal, which were substituted by ß(1-3) linked Gal, at δ 4.171 and it had the NAc shifts of ß(1-6) linked GlcNAc at δ 2.063 and 2.044. From these observations it was concluded that the decasaccharides in TQM-1-2-2 consisted of lacto-N-novooctaose cores with a ß(1-3) linked Gal and a non reducing α(2-3) linked Neu5Ac.

TQM-1-2-4

The MALDI-TOF MS of the oligosaccharides in TQM-1-2-4 had the MS ions at 2253 and 2291, of [M+K] and [M+2K-H], respectively, indicating a monosaccharide composition of $[Neu5Ac]_1[Hex]_8[HexNAc]_3$.

The ^{1}H-NMR spectrum (Fig. 8, chemical shifts in Supplemental Table 3) had H-3 axial, H-3 equatorial and NAc shifts at δ 1.801, 2.760 and 2.030, respectively, and H-3 of ß-Gal, which was substituted by α(2-3) linked Neu5Ac at δ 4.121, indicating the presence of a Neu5Ac(α2-3)Gal unit. It had the anomeric shifts of α-Glc, internal ß(1-3) linked Gal, ß-Glc, two ß(1-6) linked GlcNAc, external ß(1-3) linked Gal and ß(1-6) linked GlcNAc at δ 5.230, 4.687, 4.672, 4.649 and 4.644, 4.619 and 4.591, respectively. The spectrum had the H-1 of ß(1-4) linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.553, the H-1 of ß(1-4) linked Gal, which was substituted by ß(1-3) linked Gal, at δ 4.523, 4,506 and 4.503, and the H-1 of external ß(1-4) linked Gal at δ 4.476 and 4.470. It had the H-4 of ß(1-3) or ß(1-4) linked Gal, which were substituted at OH-3 by ß(1-3) linked Gal, at δ 4.172, 4.166 and 4.155. The spectrum had the NAc shifts of ß(1-6) linked GlcNAc at δ 2.071, 2.068, 2.063, 2.044 and 2.041. The total intensity of the shifts at δ 2.071, 2.068 and 2.063 was twice as great as that of the shifts at δ 2.044 and 2.041, similar to those of TQM-2-3.

These observations suggested that the oligosaccharides in TQM-1-2-4

contain neutral oligosaccharide units similar to the oligosaccharides in TQM-2-3 as well as non reducing α(2-3)Neu5Ac linked to Gal(ß1-3)Gal or to either of the Gal(ß1-4)GlcNAc units, as shown in Fig. 9.

Discussion

In our previous studies the neutral oligosaccharides were usually separated from the acidic ones by ion exchange chromatography and then purified by HPLC using a Hypercarb carbon column, while the acidic oligosaccharides were purified by normal phase HPLC with an Amide-80 column [11-15]. In this study, however, the neutral saccharide TQM-2-3 was purified by HPLC with an Amide-80 column. Even though we assumed that fraction TQM-2 (see Fig. 1b) contained mainly acidic oligosaccharides prior to the purification by HPLC of TQM-2-1 to TQM-2-4 (see Fig. 5a), a major component, TQM-2-3, was a neutral saccharide.

Table 1 shows that most of the previously characterized oligosaccharides of eastern quoll milk and those of other marsupials were found in this tiger quoll milk carbohydrate as well [15]. The predominant saccharides in the tiger quoll milk carbohydrate were the branched oligosaccharides lacto-N-novopentaose I (TQM-5-1 and TQM-5-2) and lacto-N-novooctaose (TQM-3-1and TQM-3-2), illustrating that branched saccharides predominate over the linear. In this regard, the tiger quoll milk oligosaccharides closely resembled those of the eastern quoll milk [15]. In milks of other, non-carnivorous marsupials including the tammar wallaby [5-9], red kangaroo [11], brushtail possum [12], koala [13] and wombat [14], linear oligosaccharides predominate over the branched ones.

In this study, the larger oligosaccharides TQM-2-3 and TQM-1-2-4, which contain three Gal(ß1-4)GlcNAc(ß1-6) units, are the first to have been identified among any marsupial milks. However, we assumed that the undecasaccharide in TQM-2-3 would be similar to that in DV-2-9 and DV-2-10 of eastern quoll milk, because their MS ions were similar [15]. It can be hypothesized that, during the biosynthesis of the undecasaccharides (Fig. 7, enclosed structures in the frame), a Gal residue is attached, via a ß(1-3)

linkage, to a non reducing Gal of a Gal(β1-4)GlcNAc unit of lacto-N-novooctaose, after which GlcNAc gets bound to the penultimate Gal, via a β(1-6) linkage, followed by another Gal being attached to the GlcNAc via a β(1-4) linkage. The biosynthesis of these oligosaccharides would presumably involve at least three glycosyltransferases known to be present in the mammary glands of lactating tammar wallabies, viz β3- and β4-galactosyltransferases and a β6-N-acetylglucosaminyltransferase [20, 21].

It seems likely that the tiger quoll milk carbohydrate contains oligosaccharides that are even larger than the undecasaccharide and the sialyl undecasaccharide because the HPLC of TQM-1-1 using a Hypercarb carbon column yielded several peaks such as TQM-1-1-1 to TQM-1-1-4 (see Fig. 3c) while that of TQM-1-2 using the Amide-80 column revealed later eluted peaks such as TQM-1-2-5 to TQM-1-2-15 (see Fig. 5b). It is therefore possible that the undecasaccharides may be further elongated by addition of β(1-3) linked Gal and Gal(β1-4)GlcNAc and that such megasaccharides could be synthesized by this elongation. It is unknown whether such larger saccharides have specific biological functions for the pouch young but, as previously noted [5] oligosaccharides exert a lower osmotic effect than do lactose or monosaccharides, and the osmotic effect of these megasaccharides may be almost negligible. This would allow the milk to contain higher concentrations of electrolytes, as has indeed been found in milk of the eastern quoll [22, 23]. It is also unclear whether the milk of other, non-carnivorous marsupials contains similar megasaccharides.

Sulphated oligosaccharides were not found in tiger quoll milk carbohydrates but it is possible that they were present in the unidentified peaks in TQM-1-2 (see Fig. 5b).

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Fig. 1 Gel chromatogram (Fig. 1a) of the carbohydrate fraction of the tiger quoll milk on a BioGel P-2 column (2.5 × 100 cm). For details, see Methods. Each fraction was monitored by the phenol-H_2SO_4 method at 490 nm (solid line) and the periodate – resorcinol method at 630 nm (dotted line). The aliquots of the first eluted peak fraction (as shown in TQM-1 ~ TQM-3) were diluted with four volumes of distilled water and were monitored as shown in Fig. 1b.

Fig. 2 High performance liquid chromatograms with a Hypercarb column of (a) fraction TQM-8 and (b) fraction TQM-5 which had been separated from the carbohydrate fraction of tiger quoll milk by gel chromatography (see Fig. 1a). For HPLC conditions, see Methods.

Fig. 3 High performance liquid chromatograms with a Hypercarb carbon column of (a) fraction TQM-4, (b) fraction TQM-3 and (c) fraction TQM-1-1. TQM-4 and TQM-3 had been separated from the carbohydrate fraction of tiger quoll milk by gel chromatography (see Fig. 1a), while TQM-1-1 had been separated by gel chromatography (see Fig.1b), followed by ion exchange chromatography (see Fig. 4b). For details, see Methods.

Fig. 4 The anion exchange chromatograms of (a) TQM-2 and (b) TQM-1 separated from the carbohydrate fraction of tiger quoll milk by gel chromatography (see Fig. 1b). For details of the chromatography conditions, see Methods. Each fraction was monitored by the phenol-H_2SO_4 method.

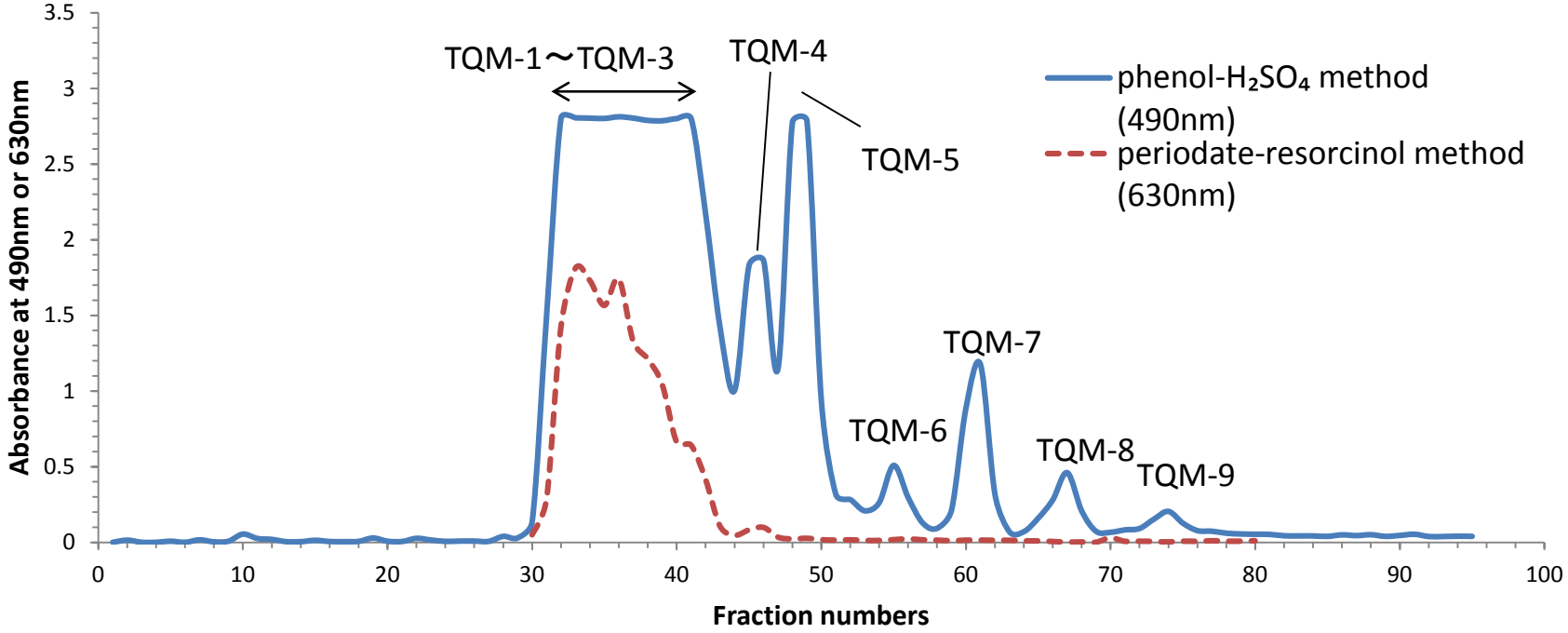
Fig. 5 High performance liquid chromatograms using a TSK-gel Amide-80 column of (a) fraction TQM-2 and (b) fraction TQM-1-2, separated by the ion exchange chromatography (see Fig. 4a and b). For HPLC conditions, see Methods.

Fig. 6 ^{1}H-NMR spectrum of oligosaccharides in TQM-2-3 isolated from tiger quoll milk carbohydrate by HPLC (see Fig. 5a). The spectrum was obtained in D_2O at 600 MHz with a Varian INOVA-600 Fourier transform – NMR spectrometer operated at 293.1 K.

Fig. 7 Three possible structures of the oligosaccharides in TQM-2-3. The two structures enclosed in frames are considered to be more plausible than the third one.

Fig. 8 ^{1}H-NMR spectrum of oligosaccharides in TQM-1-2-4 isolated from tiger quoll milk carbohydrate by HPLC (see Fig. 5b). The spectrum was obtained in D_2O at 600 MHz with a Varian INOVA-600 Fourier transform – NMR spectrometer operated at 293.1 K.

Fig. 9 Structures of the acidic oligosaccharides characterized in this study.


TQM-1～TQM-3
TQM-4
TQM-5
TQM-6
TQM-7
TQM-8
TQM-9
phenol-H₂SO₄ method (490nm)
periodate-resorcinol method (630nm)
Absorbance at 490nm or 630nm
Fraction numbers

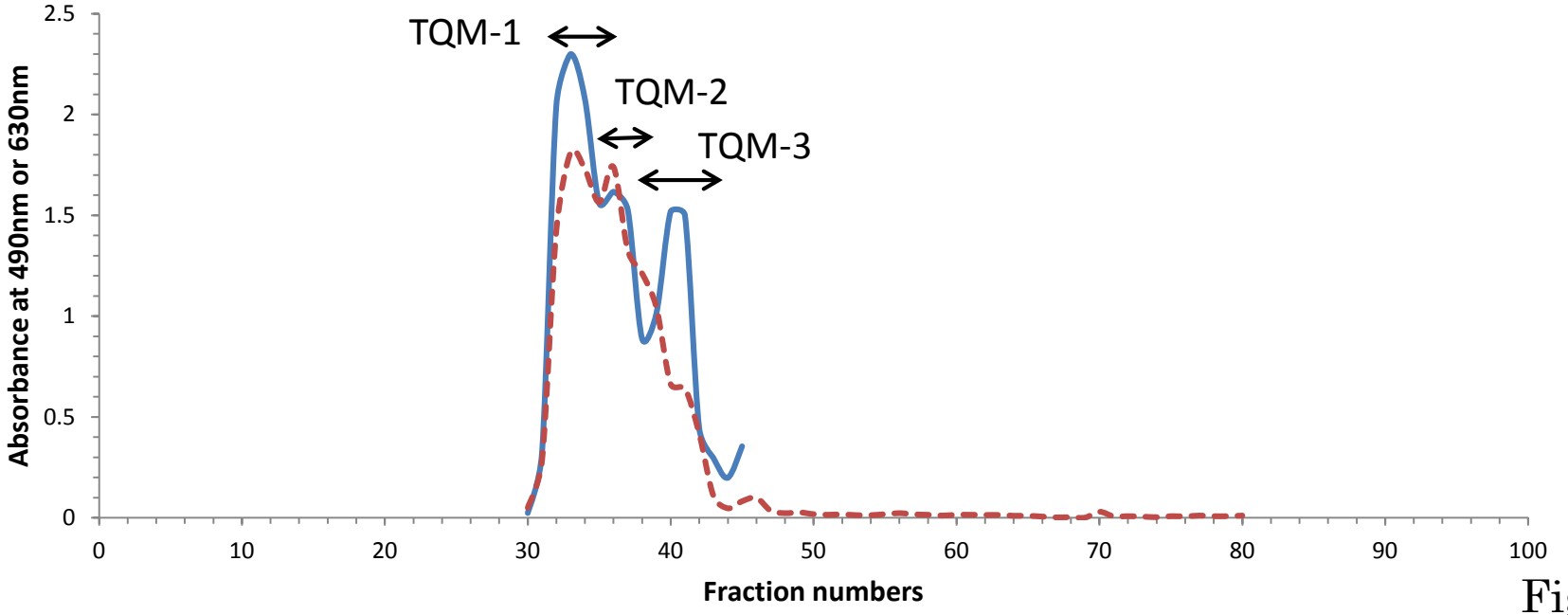
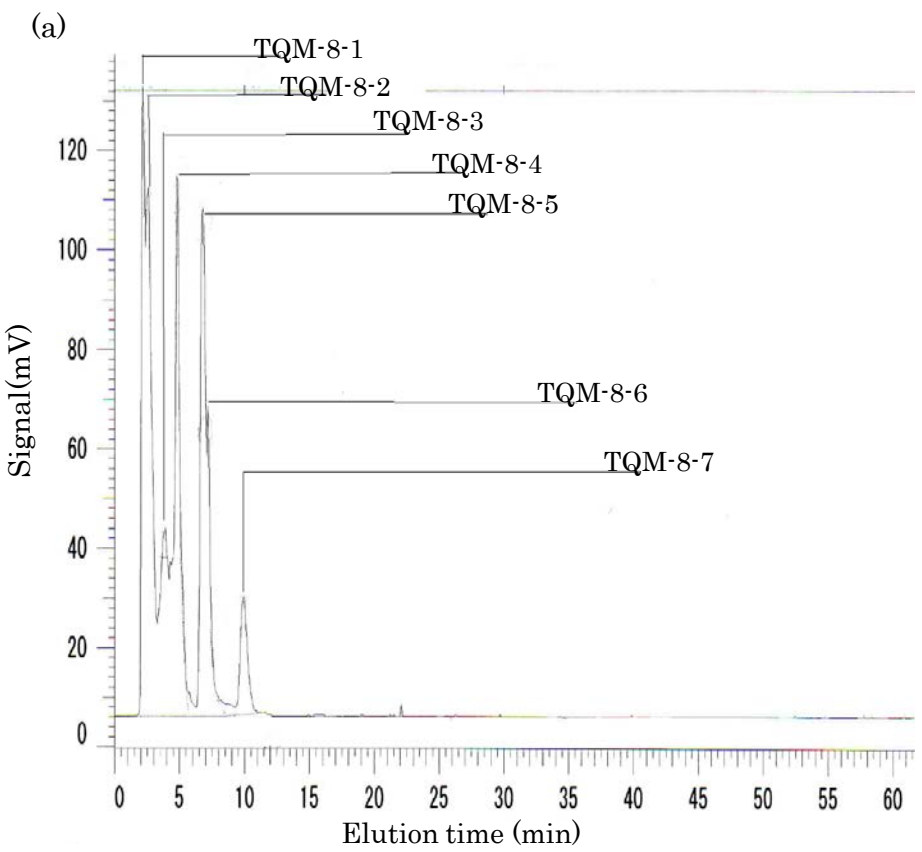

TQM-1
TQM-2
TQM-3
Absorbance at 490nm or 630nm
Fraction numbers

Fig. 1


(a)
TQM-8-1
TQM-8-2
TQM-8-3
TQM-8-4
TQM-8-5
TQM-8-6
TQM-8-7
Signal(mV)
Elution time (min)

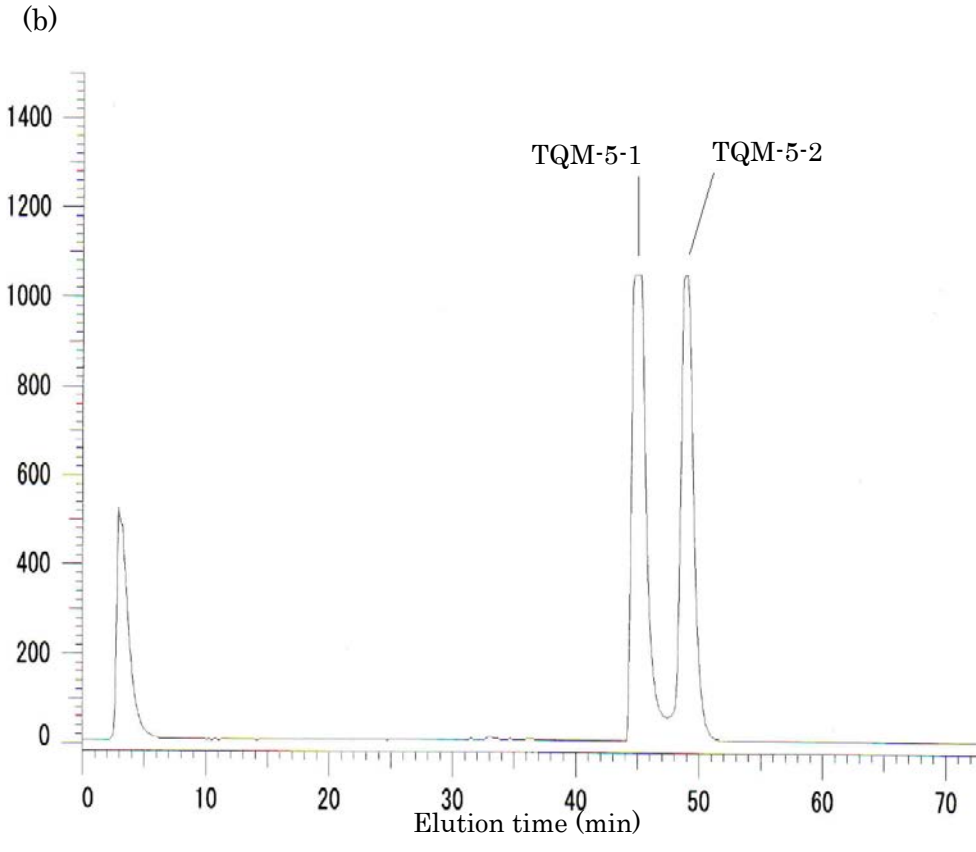

(b)
TQM-5-1
TQM-5-2
Elution time (min)

Fig. 2

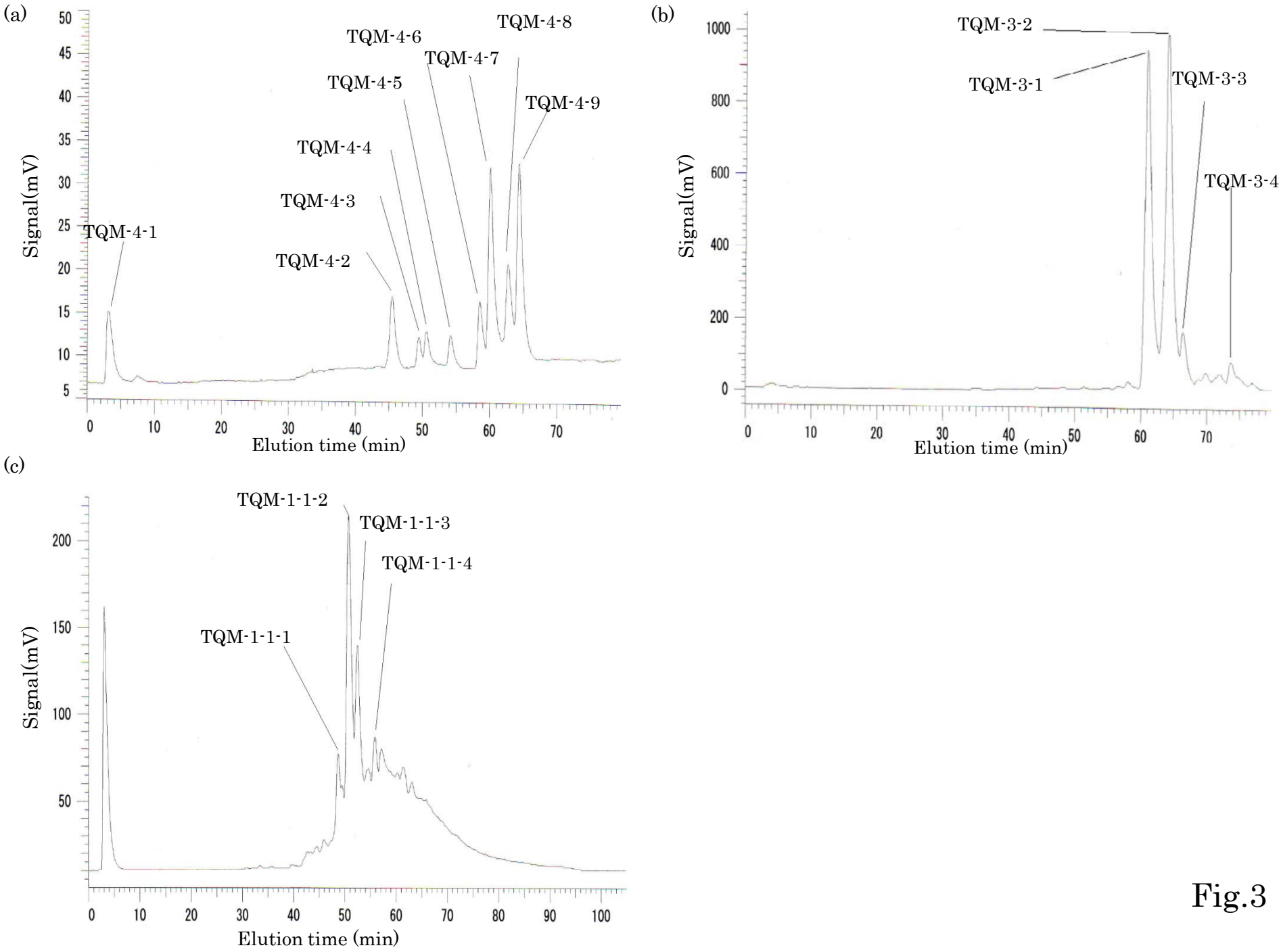

(a)
TQM-4-1
TQM-4-2
TQM-4-3
TQM-4-4
TQM-4-5
TQM-4-6
TQM-4-7
TQM-4-8
TQM-4-9
Signal(mV)
Elution time (min)
(b)
TQM-3-1
TQM-3-2
TQM-3-3
TQM-3-4
Signal(mV)
Elution time (min)
(c)
TQM-1-1-1
TQM-1-1-2
TQM-1-1-3
TQM-1-1-4
Signal(mV)
Elution time (min)

Fig.3

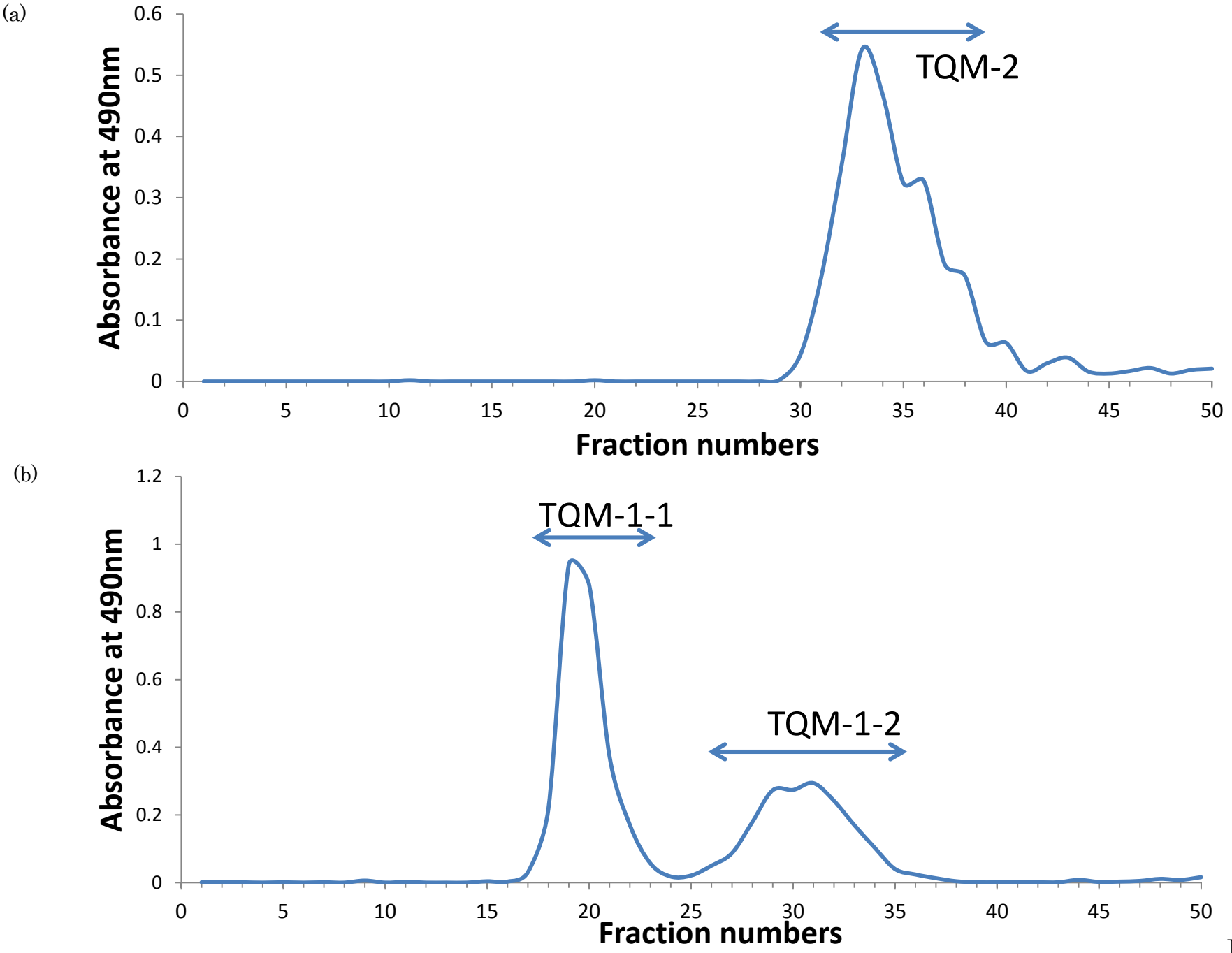

(a)
Absorbance at 490nm
0.6
0.5
0.4
0.3
0.2
0.1
0
TQM-2
0
5
10
15
20
25
30
35
40
45
50
Fraction numbers
(b)
Absorbance at 490nm
1.2
1
0.8
0.6
0.4
0.2
0
TQM-1-1
TQM-1-2
0
5
10
15
20
25
30
35
40
45
50
Fraction numbers

Fig. 4

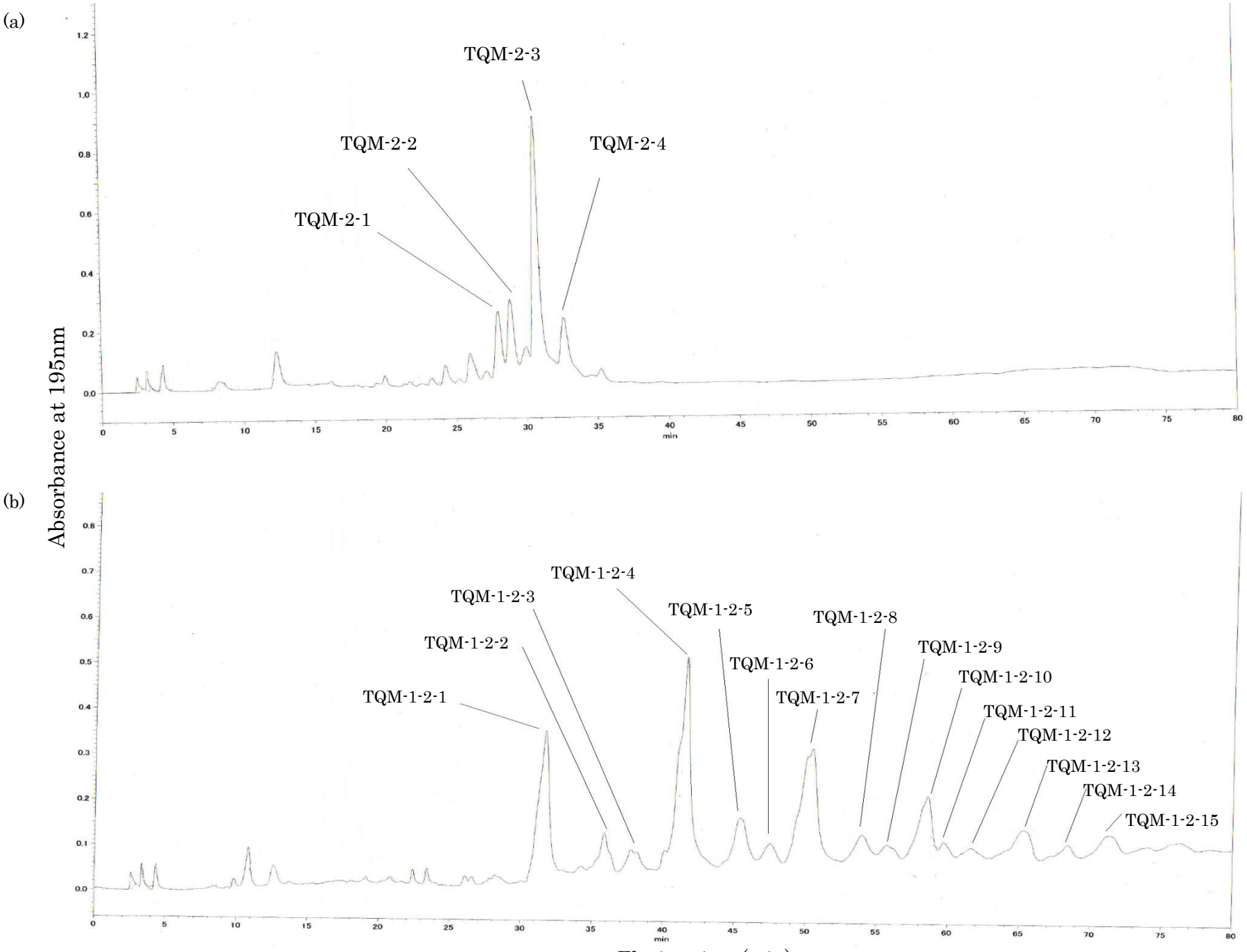

(a)
TQM-2-3
TQM-2-2
TQM-2-4
TQM-2-1
(b)
TQM-1-2-4
TQM-1-2-3
TQM-1-2-5
TQM-1-2-8
TQM-1-2-2
TQM-1-2-9
TQM-1-2-6
TQM-1-2-10
TQM-1-2-1
TQM-1-2-7
TQM-1-2-11
TQM-1-2-12
TQM-1-2-13
TQM-1-2-14
TQM-1-2-15
Absorbance at 195nm
Elution time (min)
min

Fig.5

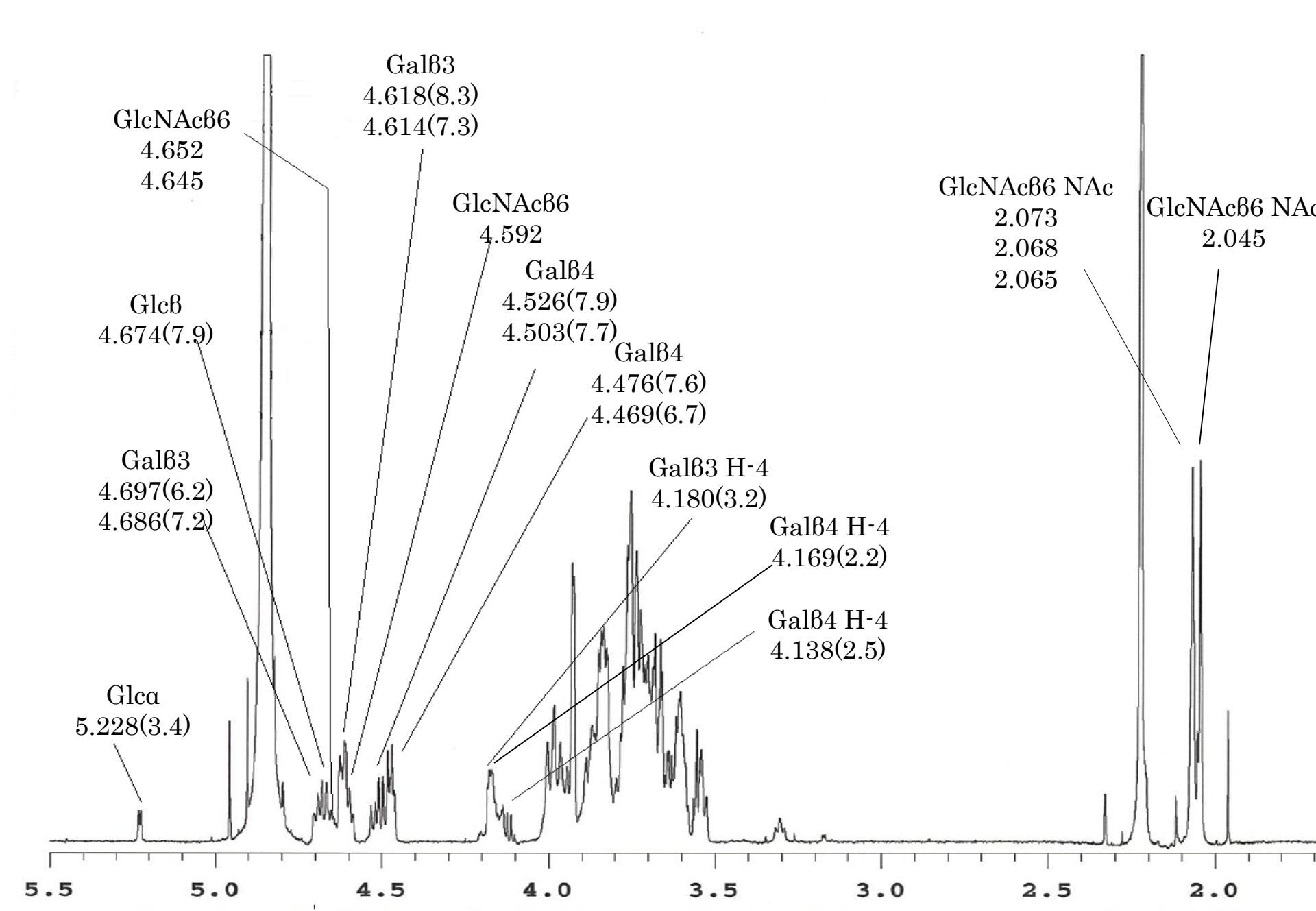

Galß3
4.618(8.3)
4.614(7.3)
GlcNAcß6
4.652
4.645
GlcNAcß6
4.592
Galß4
4.526(7.9)
4.503(7.7)
Glcß
4.674(7.9)
Galß4
4.476(7.6)
4.469(6.7)
Galß3
4.697(6.2)
4.686(7.2)
Galß3 H-4
4.180(3.2)
Galß4 H-4
4.169(2.2)
Galß4 H-4
4.138(2.5)
Glcα
5.228(3.4)
GlcNAcß6 NAc
2.073
2.068
2.065
GlcNAcß6 NAc
2.045
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Chemical shifts (ppm)

Fig. 6

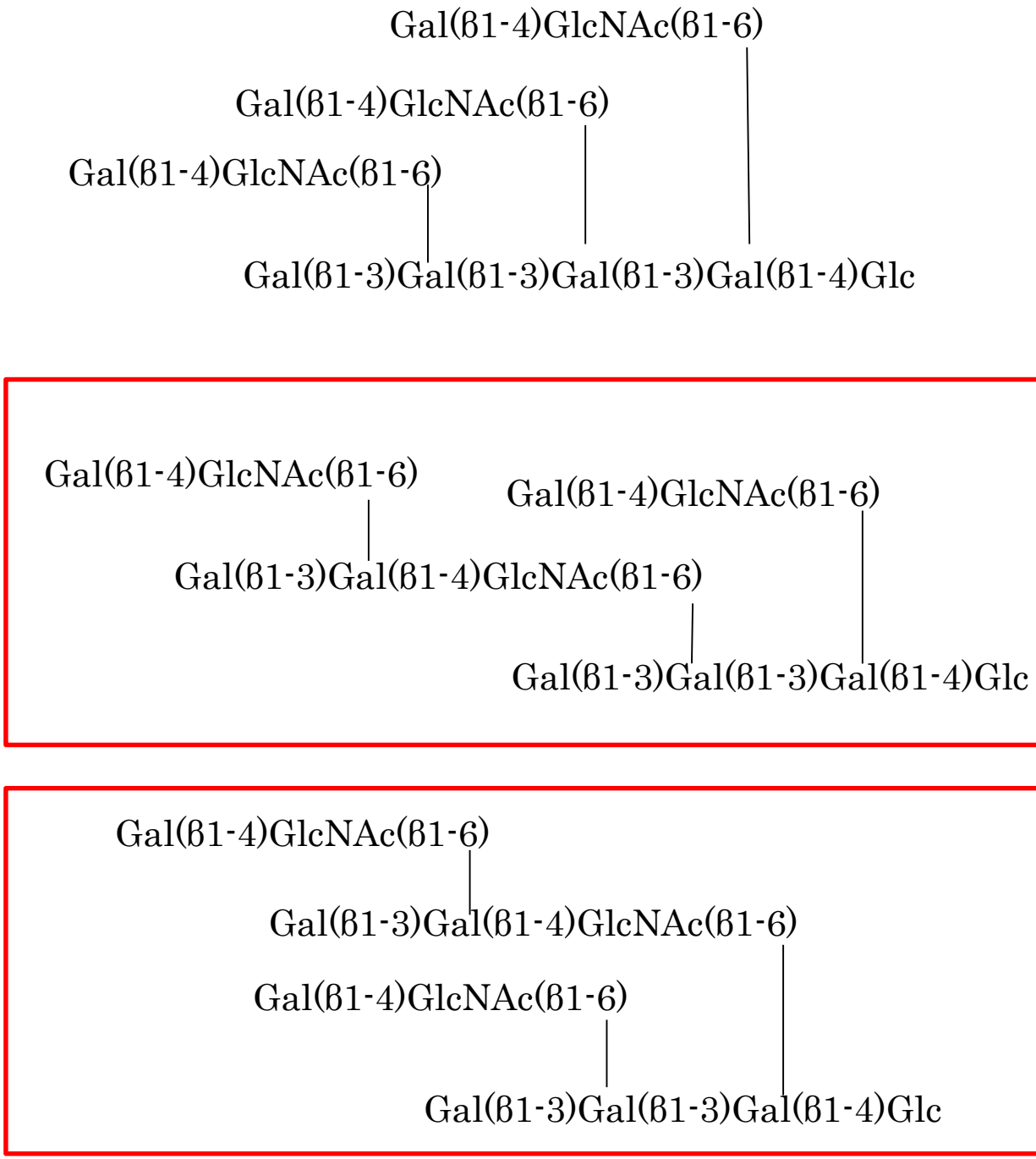

Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-3)Gal(β1-4)GlcNAc(β1-6)
Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-3)Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)GlcNAc(β1-6)
Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

Fig. 7

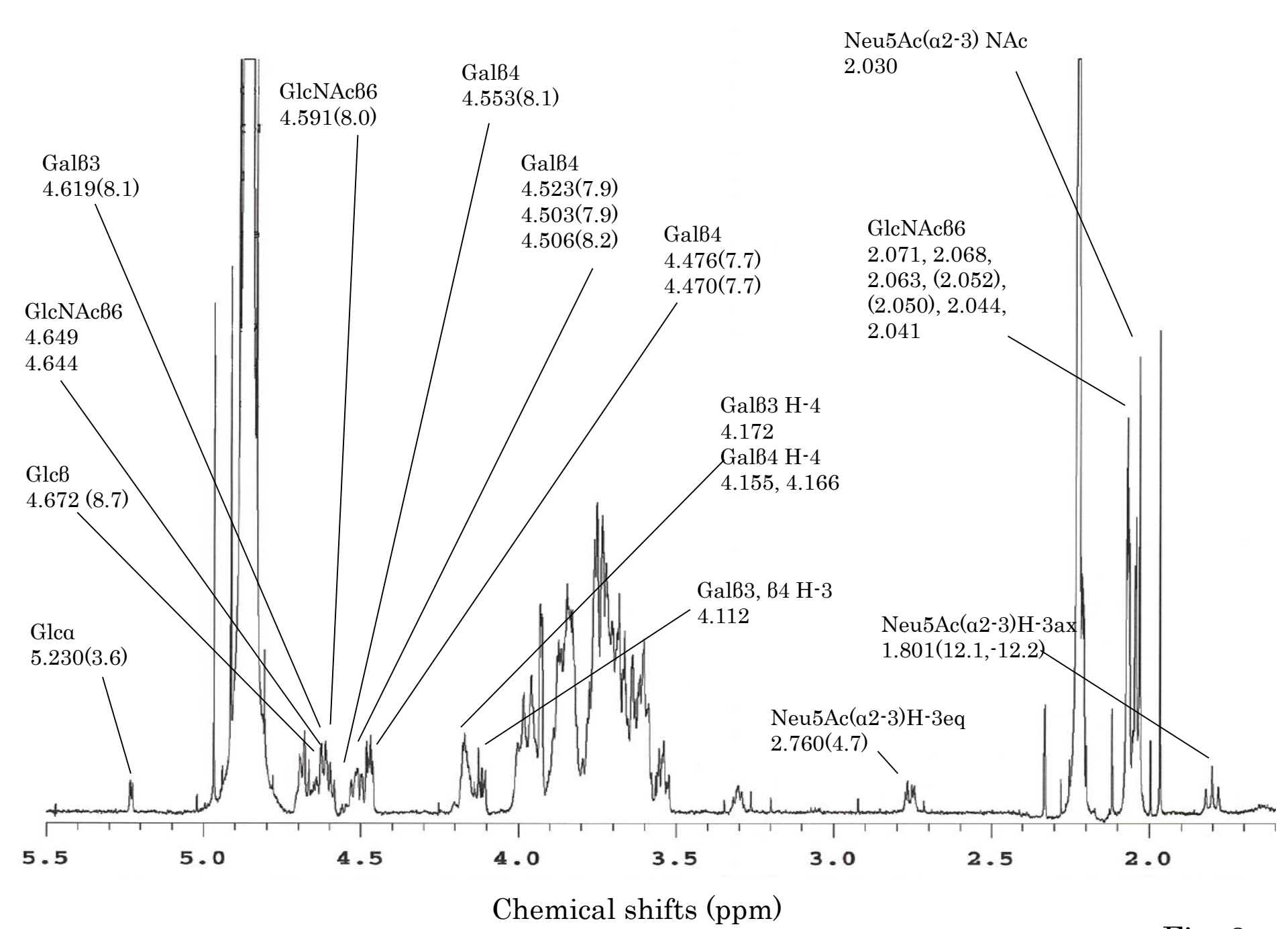
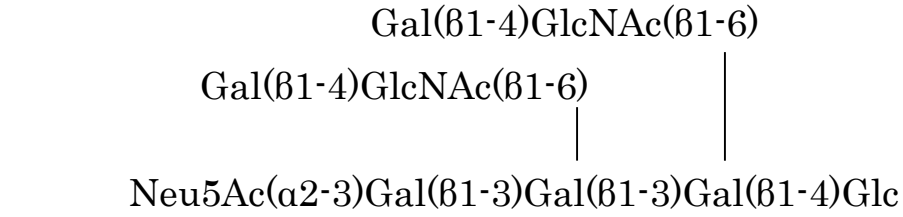
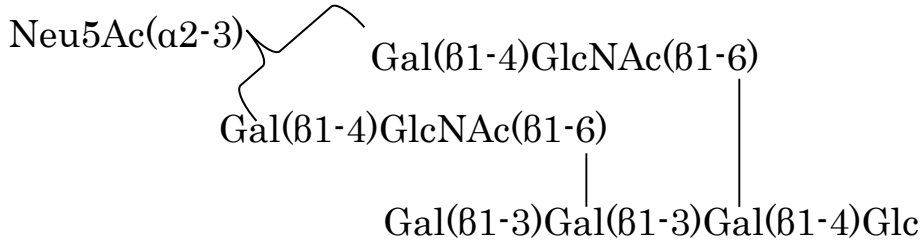

Neu5Ac(α2-3) NAc
2.030
Galβ4
4.553(8.1)
GlcNAcβ6
4.591(8.0)
Galβ3
4.619(8.1)
Galβ4
4.523(7.9)
4.503(7.9)
4.506(8.2)
Galβ4
4.476(7.7)
4.470(7.7)
GlcNAcβ6
2.071, 2.068,
2.063, (2.052),
(2.050), 2.044,
2.041
GlcNAcβ6
4.649
4.644
Galβ3 H-4
4.172
Galβ4 H-4
4.155, 4.166
Glcβ
4.672 (8.7)
Galβ3, β4 H-3
4.112
Glcα
5.230(3.6)
Neu5Ac(α2-3)H-3ax
1.801(12.1,-12.2)
Neu5Ac(α2-3)H-3eq
2.760(4.7)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Chemical shifts (ppm)

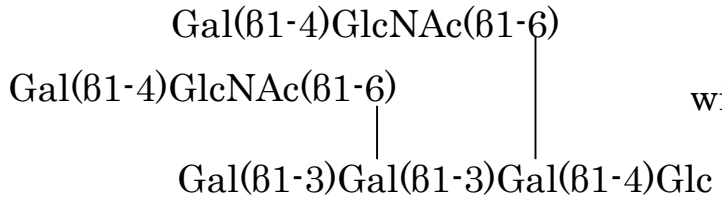
Fig. 8



TQM-1-2-1

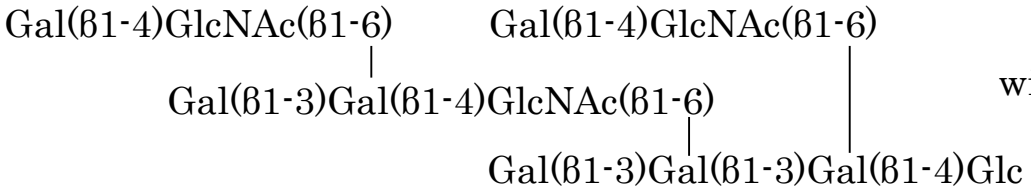


TQM-1-2-1



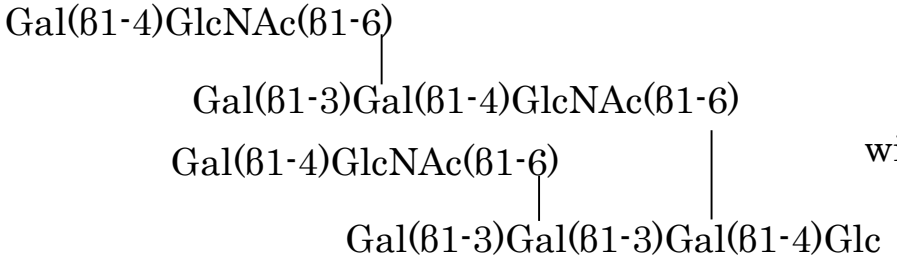
TQM-1-2-2

with a Gal(β1-3) and a Neu5Ac(α2-3)



TQM-1-2-4

with a Neu5Ac(α2-3)



TQM-1-2-4

with a Neu5Ac(α2-3)

Fig. 9

Table 1 Structures of tiger quoll milk oligosaccharides previously found in other marsupial milks

Fraction	Name	Structure
TQM-8-4 TQM-8-5	lac	Gal(β1-4)Glc
TQM-7	3'-GL	Gal(β1-3)Gal(β1-4)Glc
TQM-6	3'',3'-dGL	Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc
TQM-5-1 TQM-5-2	novoLNP Ⅰ	Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc
TQM-4-4 TQM-4-5	GalnovoLNP Ⅱ	Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc
TQM-4-6 TQM-4-8	GalnovoLNP Ⅰ	Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc
TQM-4-7	GalnovoLNPⅢ	Gal(β1-3)[Gal(β1-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc
TQM-3-1	novoLNO	Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc

Abbreviation : *lac* lactose, *3'GL* 3'-galactosyllactose, *3'',3'-dGL* 3'', 3'-digalactosyllactose, *novoLNP Ⅰ* lacto-N-novopentaose Ⅰ, *GalnovoLNP Ⅰ* galactosyllacto-N-novopentaose Ⅰ, *GalnovoLNP Ⅱ* galactosyllacto-N-novopentaose Ⅱ, *GalnovoLNP Ⅲ* galactosyllacto-N-novopentaose Ⅲ, *novoLNO* lacto-N-novooctaose