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Introduction of a new scale into reversed-phase high-performance liquid chromatography of pyridylamino sugar chains for structural assignment

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Abstract

Addition of a monosaccharide residue to a pyridylaminated (PA)-N-linked sugar chain results in an increment or decrement in the elution time on reversed-phase HPLC, the difference being defined as the partial elution time of the residue. Based on this principle, an empirical rule was deduced, which states that the elution time is roughly equal to the sum of the partial elution times of the component sugar residues [Anal. Biochem., 167 (1987) 321–326]. In practice, however, some partial elution times obtained from different pairs of mother PA-sugar chains are found to deviate, and consequently the closeness of the elution times of PA-sugar chains calculated therefrom to the observed times is reduced in such cases. To improve the reliability of the additivity rule and to generalize elution times so that they are less dependent on minor alterations in the elution conditions, we have devised a new scale for elution time, which we have named a reversed-phase scale. The elution times on the reversed-phase scale (the *R* values) are read from a conversion curve constructed using the elution times of eight selected standard PA-sugar chains. The partial elution times on the reversed-phase scale of 22 monosaccharide residues were calculated from the *R* values of 93 PA-sugar chains. The *R* values obtained by summing the partial elution times of all the component monosaccharide residues became much closer to the *R* values obtained from the reversed-phase scale, compared to the results obtained using the previous method. In addition, the *R* values were less influenced by minor change in the elution conditions. These features of the new scale allow more accurate structural assignment of sugar chains. © 1998 Elsevier Science B.V.

Keywords: Reversed-phase scale; Structure analysis; Sugars; Pyridylaminosugar chains

1. Introduction

Structures of sugar chains are usually determined by a combination of chemical and physicochemical techniques, including methylation analysis, periodate oxidation, exoglycosidase digestion, nuclear magnetic resonance spectroscopy, and mass spectroscopy. When such methods cannot be utilized because the amount of a sample is limited, a combination of tagging a sugar chain with a sensitively detectable group and its chromatographic analysis is a powerful means of analyzing the chain structure (reviewed in [1–5]). Identification of fluorogenic PA-sugar chains by two different separation principles – reversed-phase HPLC and size-fractionation HPLC (two-dimensional mapping) – has been effectively applied

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to the structure analysis of N-and O-linked sugar chains [6–9] by virtue of the fact that reversed-phase HPLC elution times reveal structural information, including linkage positions, sugar types, and anomericity.

An empirical additivity rule concerning reversed-phase HPLC elution times was deduced by Hase et al. [6,10], and by Lee et al. [11]. Based on the principle that the addition of a monosaccharide residue to a PA-sugar chain causes an increment or decrement in the elution time, the difference being defined as the partial elution time of the residue, the elution time of a PA-sugar chain was found to be roughly equal to the sum of the partial elution times of all the component monosaccharide residues. This additivity rule implies the possibility that the elution time of a PA-sugar chain can be estimated even if the standard sugar chain with the same structure is not to hand. However, because partial elution times are somewhat dependent on those of the mother PA-sugar chains from which they are calculated, the smaller the deviation in the partial elution times becomes, the more precisely can the elution time be predicted.

Taking account of the fact that deviations in partial elution times are mainly due to the mode of gradient elution, we have developed a means of manipulating reversed-phase HPLC elution times to realize a more accurate prediction of elution times from chain structures.

2. Materials and methods

2.1. Materials

GN (standard a) and GN2 (standard c) (the structures of the PA-sugar chains used in this study and their abbreviations are listed in Table 1) were prepared by reductive amination of GlcNAc and GlcNAcβ1-4GlcNAc, respectively [12]. M2X, TE, TEF3′, TR1.2.3, TR1.2.3F3′, BI, and HYB [8]; M1A, M2A, M2B, M3A, M3B (standard e), M3C, M4A, M4B, M4C, and M5A [13]; M6A, M6B, M6C, M7A, M7B, M7C, M7D, M8A, M8B, M8C, and M9A [6]; MX, MF, MFX, M2FX, M3X, M3FX, and M4X [14]; GN2F3 and GN2F6 (standard d) [15]; MO1 and MO2 [16]; and AG2BSF and AG1.2BSF [17] were prepared as described previously. TE-G3, TE-G4, and M3F6 (standard f) were purchased from Nakano Vinegar (Nagoya, Japan); AG1, AG2, AG3, AG4, AG1.2, AG1.3, AG1.4, AG2.3, AG2.4, AG3.4, AG1.2.3, AG1.2.4, AG1.3.4, AG2.3.4, AG1.2.3.4, TEF6, and TR1.2.3F were from Takara Biomedicals (Kyoto, Japan). TR1.2.4F was kindly donated by Dr. M. Oh-eda (Chugai Pharmaceutical Co., Tokyo, Japan). BIF and BIBSF (standard h) were prepared from human IgG [18], M5GN from hen egg ovalbumin [19], and M60 from ricin B chain [20]. M5B was prepared from M6B by digestion with α-mannosidase (Japanese quail oviduct) [21]. BI-G1 and BI-G2; BIF-G1, BIF-G2, and AG1.2F; AG1.2.3F; AG1.2.4F; AG1.2.3.4F; and BIBSF-G2 were prepared by digestion with β-galactosidase (*Aspergillus oryzae*) of the corresponding PA-sugar chains (BI; BIF; TR1.2.3F; TR1.2.4F; TEF6; and BIBSF, respectively). MO1F, MO2F, M5BS, MO1BSF, and GNF6 (standard b) were obtained from BIF-G2, BIF-G1, HYB, BIBSF-G2, and GN2F6, respectively, and AG1BSF and M3BSF from AG1.2BS, by digestion with β-*N*-acetylhexosaminidase (jack bean). TR1.2.4, AG1.2BS, AG2BS, AG1BS, M3BS, and BIBS (standard g) were prepared from TR1.2.4F, AG1.2BSF, AG2BSF, AG1BSF, M3BSF, and BIBSF, respectively, by digestion with α-fucosidase (bovine epidymis). AG1F and AG2F were prepared by digestion with β-galactosidase (*Aspergillus oryzae*) from MO1F and MO2F, respectively. Cosmosil 5C18 P was purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Reversed-phase HPLC

A Nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) was used. Reversed-phase HPLC was done with a Cosmosil 5C18 P column (250×1.5 mm) at a flow-rate of 150 μl min^{-1} at 25°C. The column was equilibrated with 20 m*M* ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. After injection of a sample, the concentration of 1-butanol was increased linearly to 0.4% over 90 min. The elution was monitored by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

Structures of PA-sugar chains used and their abbreviations

Abbreviation	Structure
GN (a)	GlcNAc–PA
GNF6 (b)	Fucα1–6GlcNAc–PA
GN2 (c)	GlcNAcβ1–4GlcNAc–PA
M1A	Manβ1–4GlcNAcβ1–4GlcNAc–PA
M2A	Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M2B	Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA
M3A	Manα1–6 Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA
M3B (e)	Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M3C	Manα1–3Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA
M4A	Manα1–6 Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M4B	Manα1–6 Manα1–3Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA
M4C	Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M5A	Manα1–6 Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M5B	Manα1–3Manα1–6 Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M6O	Manα1–3Manα1–6 Manα1–2Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M6A	Manα1–2Manα1–6 Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M6B	Manα1–6 Manα1–3Manα1–6 Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M6C	Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M7A	Manα1–2Manα1–6 Manα1–3Manα1–6 Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M7B	Manα1–6 Manα1–3Manα1–6 Manα1–2Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M7C	Manα1–2Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M7D	Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M8A	Manα1–2Manα1–6 Manα1–3Manα1–6 Manα1–2Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M8B	Manα1–2Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M8C	Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–2Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA

(Cont.)

Table 1. Continued

Abbreviation	Structure
M9A	Manα1–2Manα1–6 Manα1–2Manα1–3Manα1–6 Manα1–2Manα1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
GN2F3	GlcNAcβ1–4GlcNAc–PA Fucα1–3
GN2F6 (d)	Fucα1–6 GlcNAcβ1–4GlcNAc–PA
MX	Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2
MF	Manβ1–4GlcNAcβ1–4GlcNAc–PA Fucα1–3
MFX	Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2 Fucα1–3
M2X	Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2
M2FX	Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2 Fucα1–3
M3X	Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2
M3F6 (f)	Manα1–6 Fucα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
M3FX	Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Xylβ1–2 Fucα1–3
M4X	Manα1–3Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc– PA Xylβ1–2
AG1	Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG2	GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG3	Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG4	GlcNAcβ1–6 Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG1.2	GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.3	Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.4	GlcNAcβ1–6 Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG2.3	GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG2.4	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA

Table 1. Continued

Abbreviation	Structure
AG3.4	GlcNAcβ1–6 Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG1.2.3	GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.2.4	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.3.4	GlcNAcβ1–6 Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG2.3.4	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG1.2.3.4	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1F	Manα1–6 Fucα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG2F	GlcNAcβ1–2Manα1–6 Fucα1–6 Manα1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA
AG1.2F	GlcNAcβ1–2Manα1–6 Fucα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.2.3F	GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.2.4F	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 Fucα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
AG1.2.3.4F	GlcNAcβ1–6 GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2
BI	Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
BI-G1	Galβ1–4GlcNAcβ1–2Manα1–6 GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
BI-G2	GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
BIF	Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
BIF-G1	Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
BIF-G2	GlcNAcβ1–2Manα1–6 Fucα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
MO1	Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA
MO2	Galβ1–4GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA

(Cont.)

Table 1. Continued

Abbreviation	Structure
MO1F	Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Fucα1–6
MO2F	Galβ1–4GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Fucα1–6
TR1.2.3	Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2
TR1.2.4	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2
TR1.2.3F	Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2 Fucα1–6
TR1.2.3F3'	Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Fucα1–3 Galβ1–4GlcNAcβ1–2
TR1.2.4F	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcb1–2 Fucα1–6
TE	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2
TE-G3	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2
TE-G4	GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2
TEF6	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2 Fucα1–6
TEF3'	Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–4Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc–PA Fucα1–3 Galβ1–4GlcNAcβ1–2
AG1BS	Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3
AG2BS	GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Manα1–3
AG1.2BS	GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3
AG1BSF	Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3 Fucα1–6

Table 1. Continued

Abbreviation	Structure
AG2BSF	GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Manα1–3
AG1.2BSF	GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3
BIBS (g)	Galβ1–4GlcNAcβ1–2Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2Manα1–3
BIBSF (h)	Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2Manα1–3
BIBSF-G2	GlcNAcβ1–2Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2Manα1–3
MO1BSF	Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Galβ1–4GlcNAcβ1–2Manα1–3
M3BS	Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Manα1–3
M3BSF	Manα1–6 Fucα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Manα1–3
M5GN	Manα1–6 Manα1–3Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3
M5BS	Manα1–6 Manα1–3Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA Manα1–3
HYB	Manα1–6 Manα1–3Manα1–6 GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc–PA GlcNAcβ1–2Manα1–3

2.3. Construction of conversion curve

A conversion curve (Fig. 1) to convert elution times (E) to reversed-phase scale values (R values) was constructed using the elution times of eight standard PA-sugar chains (E_a, E_b, E_c, E_d, E_e, E_f, E_g, and E_h, where the suffixes represent the standard PA-sugar chains indicated in parentheses in Table 1). The conversion curve is comprised of seven linear sections: L_1, L_2, L_3, L_4, L_5, L_6, and L_7.

L_1: $R=pE/(E_b-E_a)$, $(E\leq E_b)$

L_2: $R=A_1E+B_1$, $(E_b<E\leq E_c)$

L_3: $R=pE/(E_d-E_c)+q_2$, $(E_c<E\leq E_d)$

L_4: $R=A_2E+B_2$, $(E_d<E\leq E_e)$

L_5: $R=pE/(E_f-E_e)+q_3$, $(E_e<E\leq E_f)$

L_6: $R=A_3E+B_3$, $(E_f<E\leq E_g)$

L_7: $R=pE/(E_h-E_g)+q_4$, $(E>E_g)$

where

$p=80/S_1+S_2+S_3+S_4)$

$q_2=80S_1/S_1+S_2+S_3+S_4)$

$q_3=80(S_1+S_2)/S_1+S_2+S_3+S_4)$

$q_4=80(S_1+S_2+S_3)/S_1+S_2+S_3+S_4)$

$S_1=(E_b+E_c)[(E_d-E_c)-(E_b-E_a)]/2[(E_b-E_a)(E_d-E_c)]$

$S_2=(E_d+E_e)[(E_f-E_e)-(E_d-E_c)]/2[(E_d-E_c)(E_f-E_e)]$

$S_3=(E_f+E_g)[(E_h-E_g)-(E_f-E_e)]/2[(E_f-E_e)(E_h-E_g)]$

$S_4=E_h/(E_h-E_g)$

$A_1=\{p[E_c/(E_d-E_c)-E_b/(E_b-E_a)]+q_2\}/(E_c-E_b)$

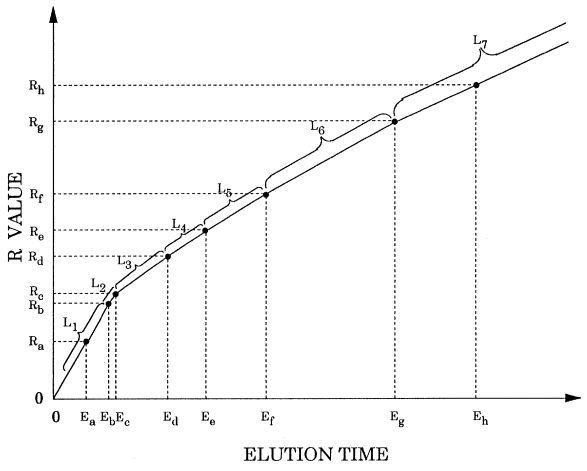

Fig. 1. Conversion curve constructed using the elution times of eight standard PA-sugar chains. E_a..... E_h, elution times of standard sugar chains a..... h, respectively; R_a..... R_h, elution times on the reversed-phase scale of standard sugar chains a..... h, respectively; L_1.... L_8, see text.

$$A_2=\{p[E_e/(E_f-E_e)-E_d/(E_d-E_c)]+q_3-q_2\}/(E_e-E_d)$$

$$A_3=\{p[E_g/(E_h-E_g)-E_f/(E_f-E_e)]+q_4-q_3\}/(E_g-E_f)$$

$$B_1=pE_b/(E_b-E_a)-A_1E_b$$

$$B_2=pE_d/(E_d-E_c)+q_2-A_2E_d$$

$$B_3=pE_f/(E_f-E_e)+q_3-A_3E_f$$

L_1 and L_3, L_3 and L_5, and L_5 and L_7 intersect at $(E_b+E_c)/2$, $(E_d+E_e)/2$, and $(E_f+E_g)/2$, respectively.

E_a, E_b, E_c, E_d, E_e, E_f, E_g, and E_h were converted to R_a, R_b, R_c, R_d, R_e, R_f, R_g, and R_h (=80), respectively, on the reversed-phase scale so as to satisfy $R_b - R_a = R_d - R_c = R_f - R_e = R_h - R_g =$ constant = the partial elution time of the Fucα1-6 residue on the reversed-phase scale. The BASIC program used to calculate the *R* value is given in Fig. 2.

```
10 '*********************************************
20 'REVERSED-PHASE CONVERSION (rps)
30 '*********************************************
40 INPUT "ELUTION TIME OF STANDARD A";E1
50 INPUT "ELUTION TIME OF STANDARD B";E2
60 INPUT "ELUTION TIME OF STANDARD C";E3
70 INPUT "ELUTION TIME OF STANDARD D";E4
80 INPUT "ELUTION TIME OF STANDARD E";E5
90 INPUT "ELUTION TIME OF STANDARD F";E6
100 INPUT "ELUTION TIME OF STANDARD G";E7
110 INPUT "ELUTION TIME OF STANDARD H";E8
120 S1=(E2+E3)*(E4-E3-E2+E1)/(2*(E4-E3)*(E2-E1))
130 S2=(E4+E5)*(E6-E5-E4+E3)/(2*(E6-E5)*(E4-E3))
140 S3=(E6+E7)*(E8-E7-E6+E5)/(2*(E8-E7)*(E6-E5))
150 S4=E8/(E8-E7)
160 P=80/(S1+S2+S3+S4)
170 Q2=80*S1/(S1+S2+S3+S4)
180 Q3=80*(S1+S2)/(S1+S2+S3+S4)
190 Q4=80*(S1+S2+S3)/(S1+S2+S3+S4)
200 T1=E3/(E4-E3)-E2/(E2-E1)
210 T2=E5/(E6-E5)-E4/(E4-E3)
220 T3=E7/(E8-E7)-E6/(E6-E5)
230 A1=(P*T1+Q2)/(E3-E2)
240 A2=(P*T2+Q3-Q2)/(E5-E4)
250 A3=(P*T3+Q4-Q3)/(E7-E6)
260 B1=P*E2/(E2-E1)-A1*E2
270 B2=P*E4/(E4-E3)+Q2-A2*E4
280 B3=P*E6/(E6-E5)+Q3-A3*E6
290 INPUT "ELUTION TIME OF SAMPLE";E
300 IF E<=E2 THEN GOTO 370
310 IF E>E2 AND E<=E3 THEN GOTO 380
320 IF E>E3 AND E<=E4 THEN GOTO 390
330 IF E>E4 AND E<=E5 THEN GOTO 400
340 IF E>E5 AND E<=E6 THEN GOTO 410
350 IF E>E6 AND E<=E7 THEN GOTO 420
360 IF E>E7 THEN GOTO 430
370 R=P*E/(E2-E1): GOTO 440
380 R=A1*E+B1: GOTO 440
390 R=P*E/(E4-E3)+Q2: GOTO 440
400 R=A2*E+B2: GOTO 440
410 R=P*E/(E6-E5)+Q3: GOTO 440
420 R=A3*E+B3: GOTO 440
430 R=P*E/(E8-E7)+Q4: GOTO 440
440 PRINT "RPS VALUE OF SAMPLE =";R
450 INPUT "CONTINUE (YES-1/NO-2)";M
460 IF M=2 THEN GOTO 470 ELSE GOTO 290
470 END
```

Fig. 2. BASIC program for calculating *R* values from elution times of PA-sugar chains using elution times of the eight standard PA-sugar chains. E1....E8 in the program correspond to E_a....E_h, respectively.

3. Results and discussion

3.1. Introduction of reversed-phase scale

The PA-sugar chains were chromatographed on a Cosmosil 5C18P column as described above. Their elution times are shown in Table 2. The elution times of sugar chains with a Fucα1-6 residue linked to a reducing-end GlcNAc residue were widely distributed over those of naturally occurring PA-N-linked sugar chains (Table 2), and the partial elution time of the Fucα1-6 residue itself was relatively large among the monosaccharide residues tested. Taking advantage of these characteristics, eight PA-sugar chains were selected as standards (a–h). The *R* values

Table 2
Observed and calculated elution times, and elution times on the reversed-phase scale of PA-sugar chains

Abbreviation	E	E_{cal}	$\lvert E-E_{cal}\rvert/E\times100$	R	R_{cal}	$\lvert R-R_{cal}\rvert/R\times100$
GN (a)	6.29			14.75		
GNF6 (b)	10.33			24.23		
GN2 (c)	11.84	11.84		26.74	26.74	
M1A	18.96	17.41	8.2	33.41	34.36	2.8
M2A	18.95	18.61	1.8	33.40	35.19	5.4
M2B	28.00	25.62	8.5	41.66	41.66	0.0
M3A	27.89	27.08	2.9	41.57	42.36	1.9
M3B (e)	28.93	26.82	7.3	42.99	42.49	1.2
M3C	27.10	26.75	1.3	41.95	42.65	1.7
M4A	28.40	28.28	0.4	42.02	43.19	2.8
M4B	29.48	28.21	4.3	42.97	43.35	0.9
M4C	30.02	27.95	6.9	43.42	43.48	0.1
M5A	31.12	29.41	5.5	43.87	44.18	0.7
M5B	23.69	23.08	2.6	37.37	38.76	3.7
M60	22.33	21.37	4.3	37.55	37.59	0.1
M6A	24.27	21.68	11	37.84	37.61	0.6
M6B	26.95	24.54	8.9	40.22	39.46	1.9
M6C	36.70	34.22	6.8	48.54	48.72	0.4
M7A	19.15	16.81	12	33.13	32.89	0.7
M7B	25.01	22.83	8.7	38.51	38.29	0.6
M7C	28.03	26.49	5.5	42.85	42.15	1.6
M7D	31.77	29.35	7.6	44.40	44.00	0.9
M8A	17.41	15.10	13	31.51	31.72	0.7
M8B	24.22	21.62	11	37.80	37.43	1.0
M8C	29.95	27.64	7.7	42.87	42.83	0.1
M9A	22.04	19.91	9.7	35.84	36.26	1.2
GN2F3	7.64	3.07	60	17.72	17.19	3.0
GN2F6 (d)	21.50	23.60	10	36.23	36.22	0.0
MX	25.82	22.89	11	40.77	39.74	2.5
MF	11.66	8.64	26	26.28	24.81	5.6
MFX	15.97	14.12	12	30.59	30.19	1.3
M2X	33.28	31.10	6.6	46.14	47.04	2.0
M2FX	22.34	22.33	0.0	36.59	37.49	2.5
M3X	27.64	25.71	7.0	42.45	42.21	0.6
M3F6 (f)	40.28	38.58	4.2	52.47	51.98	0.9
M3FX	16.08	16.94	5.4	30.59	32.66	6.8
M4X	30.04	26.84	11	43.44	43.20	0.6
AG1	27.52	24.31	12	40.50	40.02	1.2
AG2	42.91	39.42	8.1	53.41	52.04	2.6
AG3	35.79	33.68	5.9	47.80	47.88	0.2
AG4	32.38	29.73	8.2	44.70	44.60	0.2
AG1.2	37.95	36.91	2.7	49.37	49.57	0.4
AG1.3	38.63	36.43	5.7	49.95	49.52	0.9
AG1.4	29.61	27.22	8.1	42.34	42.13	0.5
AG2.3	49.93	46.28	7.3	58.68	57.43	2.1
AG2.4	28.32	26.49	6.5	41.20	41.87	1.6
AG3.4	39.23	36.59	6.7	50.45	49.99	0.9
AG1.2.3	49.67	49.03	1.3	58.50	59.07	1.0

(Cont.)

Table 2. Continued

Abbreviation	E	E_{cal}	$\lvert E-E_{cal}\rvert/E\times100$	R	R_{cal}	$\lvert R-R_{cal}\rvert/R\times100$
AG1.2.4	26.92	23.98	11	39.95	39.40	1.4
AG1.3.4	41.27	39.34	4.7	52.17	51.63	1.0
AG2.3.4	35.03	33.35	4.8	46.92	47.26	0.7
AG1.2.3.4	38.76	36.10	6.9	50.05	48.90	2.3
AG1F	37.58	36.07	4.0	50.58	49.50	2.1
AG2F	53.86	51.18	5.0	62.60	61.52	1.7
AG1.2F	48.99	48.67	0.7	59.22	59.05	0.3
AG1.2.3F	61.54	60.79	1.2	67.93	68.55	0.9
AG1.2.4F	36.98	35.74	3.4	50.26	48.88	2.4
AG1.2.3.4F	50.60	47.86	5.4	59.76	58.36	2.0
BI	45.68	43.35	5.1	54.53	54.28	0.5
BI-G1	41.05	40.00	2.6	52.99	51.71	2.4
BI-G2	40.04	40.26	0.5	52.23	52.14	0.2
BIF	57.00	55.11	3.3	63.30	63.76	0.7
BIF-G1	54.93	51.76	5.8	61.70	61.19	0.8
BIF-G2	53.99	52.02	3.7	60.97	61.62	1.1
MO1	30.36	27.66	8.9	44.61	42.59	4.5
MO2	44.75	42.51	5.0	56.02	54.18	3.3
MO1F	41.44	39.42	4.9	53.61	52.07	2.9
MO2F	55.74	54.27	2.6	63.91	63.66	0.4
TRI1.2.3	57.70	57.19	0.9	65.00	65.05	0.1
TRI1.2.4	30.34	31.79	4.8	44.75	45.13	0.8
TRI1.2.3F	69.87	68.95	1.3	73.52	74.53	1.4
TRI1.2.3F3′	56.34	55.28	1.9	64.00	63.65	0.6
TRI1.2.4F	42.38	43.55	2.8	53.02	54.61	3.3
TE	44.14	45.63	3.4	54.98	55.90	1.7
TE-G3	42.42	43.91	3.5	53.71	54.63	1.7
TE-G4	42.77	44.26	3.5	53.96	54.88	1.7
TEF6	55.55	57.39	3.3	63.41	65.38	3.4
TEF3′	41.69	43.72	4.9	53.18	54.50	2.5
AG1BS	45.52	44.69	1.8	55.97	55.58	0.7
AG2BS	41.75	39.66	5.0	53.17	51.49	3.2
AG1.2BS	58.19	57.29	1.5	65.35	65.13	0.3
AG1BSF	59.55	56.45	5.2	66.19	65.06	1.7
AG2BSF	55.11	51.42	6.7	62.89	60.97	3.1
AG1.2BSF	72.51	69.05	4.8	75.13	74.61	0.7
BIBS (g)	64.91	63.73	1.8	70.52	69.84	1.0
BIBSF (h)	80.00	75.49	5.6	80.00	79.32	0.9
BIBSF-G2	76.63	72.40	5.5	77.78	77.18	0.8
MO1BSF	63.75	59.80	6.2	69.47	67.63	2.7
M3BS	27.95	27.06	3.2	41.62	41.94	0.8
M3BSF	42.14	38.82	7.9	51.60	51.42	0.4
M5GN	28.91	26.90	7.0	42.25	41.71	1.3
M5BS	22.33	20.62	7.7	36.40	36.71	0.9
HYB	37.33	38.49	3.1	49.33	49.80	1.0

Elution times (E) are relative times taking the elution time of BIBSF (g) as 80. R_{cal} was calculated by summing the averaged partial elution times of the constituent monosaccharide residues on the reversed-phase scale (Fig. 3), and E_{cal} by summing the averaged partial elution times of the constituent monosaccharide residues.

calculated from these standard chains with similar structures to the samples seemed to be less influenced by minor changes in the elution conditions, including column aging, than the elution times obtained using PA-isomalto-oligosaccharides [11]. The conversion curve was thus unequivocally determined by the elution times of the eight standards (Fig. 1).

3.2. Elution time and partial elution time on reversed-phase scale

The R values of the PA-sugar chains are listed in Table 2. The partial elution times of the component monosaccharide residues on the reversed-phase scale were calculated from the R values of the various pairs of PA-sugar chains with and without the residue, and their average values were obtained (Fig. 3). The calculated R values (R_{cal}) obtained by summing the partial elution times on the reversed-phase scale were much closer to the actual R values compared to the closeness of the corresponding sums of the calculated partial elution times (E_{cal}) to the actual elution times (E) (Table 2). The R values and resulting partial elution times are thus superior to the elution times and the partial elution times for predicting the elution times of the sugar chains shown in Fig. 3, and conversely, for estimating chain structures from elution times.

It should be noted that the monosaccharide residues X, BS, GN3, and GN4 have two or three partial elution times (Fig. 3). These residues seem to be sterically close to M3, GN1 and M5, GN1, and GN2, respectively, and to be influenced by interactions such as hydrogen bonding. Partial elution times obtained in the presence of an interacting partner would thus differ from those obtained without a partner. A similar situation may occur with other PA-sugar chains not examined here; however, the additivity rule seems to hold if the particular sugar residue has more than one value.

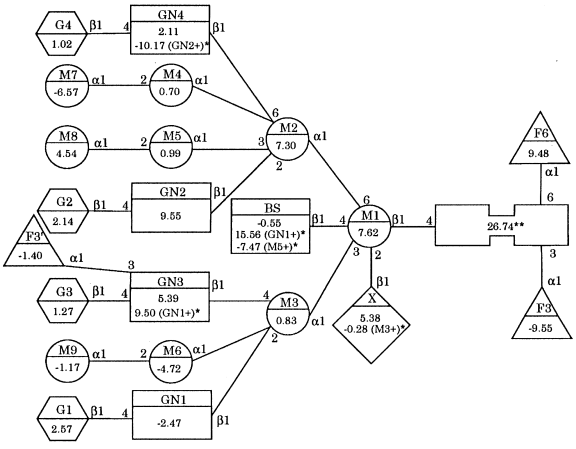

Fig. 3. Schematic representation of averaged partial elution times for monosaccharide residues of an N-linked sugar chain on the reversed-phase scale. The averaged partial elution times on the reversed-phase scale and the abbreviations of the monosaccharide residues are indicated in the symbols: ○, mannose; ◌, galactose; □, *N*-acetylglucosamine; △, fucose; ◇, xylose; □□, chitobiose. Numerals on the lines indicate linkage positions. * Values in the presence of interacting partners are indicated in parentheses. ** Partial elution time of GlcNAcβ1–4GlcNAc–PA.

Lee et al. proposed a manipulation that includes the calibration of a scale for elution time using PA-isomalto-oligosaccharides and the classification of sugar chains into four types [11]. To comply with the additivity rule, the partial elution times of monosaccharide residues vary with the chain type. In our manipulation, the reversed-phase scale was introduced to realize the additivity rule. The differences between the actual and calculated R values (R and R_{cal}) were similar in degree to those reported by Lee et al.

An experiment with a column of a different size (150×4.6 mm) showed that the R values were mostly independent of the column size. Reliable data were obtained with the same column over a period of 400 h when it was washed with methanol for 30 min after every 20 runs. Of the eight standards employed, sugar chains a, e, f, g, and h are commercially available, and b, c, and d can be easily obtained by digesting sugar chain e with exoglycosidases. The introduction of a reversed-phase scale makes two-dimensional map analysis a more powerful tool for the structural assignment of sugar chains.

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