Carbohydrate Research, 201 (1990) 125–134
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Isolation and characterization of three positional isomers of diglucosylcyclomaltoheptaose

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(Received August 1st, 1989; accepted, in revised form, October 30th, 1989)

ABSTRACT

Three positional isomers of diglucosylcyclomaltoheptaose [$(G)_2$-β-cyclodextrin], $6^1,6^4$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**1**), $6^1,6^3$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**2**), and $6^1,6^2$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**3**) were isolated by h.p.l.c. on a reversed-phase column from the mother liquors of a large-scale preparation of βCD with *Bacillus ohbensis* cyclomaltodextrin glucanotransferase (EC 2.4.1.19) and were characterized by h.p.l.c. analysis of partial hydrolyzates and by ^{13}C-n.m.r. spectroscopy. Their molecular weights were confirmed by f.a.b.-m.s. Their characteristic chromatographic behavior on four h.p.l.c. columns of different separation modes was found to be very useful for their identification. It is particularly noteworthy that the first application of a graphitized carbon column to CDs enabled a fine separation of all three positional isomers.

INTRODUCTION

Previously[1], we had isolated, from the mother liquors of a large-scale preparation of βCD, a cyclomaltoheptaose (β-cyclodextrin, βCD) having two D-glucose branches in the molecule. This βCD derivative was formed by action of *Bacillus ohbensis* cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) on potato starch, followed by hydrolysis with glucan 1,4-α-glucosidase (glucoamylase, EC 3.2.1.3) to shorten the side chains of the branched CDs. The diglucosyl-βCD [$(G)_2$-βCD] was crystallized from water, and the structure was presumed to be $6^1,6^4$-di-*O*-(α-D-glucopyranosyl)-cyclomaltoheptaose (**1**) by h.p.l.c. analysis of its partial hydrolyzates. Later detailed h.p.l.c. on reversed-phase columns[2] revealed the presence of a positional isomer **2** in the filtrate of crystalline $(G)_2$-βCD (**1**). Furthermore, during the preparative h.p.l.c. of **1** and **2** on a larger size octadecylsilyl-derivatized (ODS) column, another minor isomer **3** was also found.

In this paper we report a method of isolating all three positional isomers **1**, **2**, and **3** of $(G)_2$-βCD, their structural analyses, and their chromatographic behavior on four h.p.l.c. columns having different modes of separation.

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0008-6215/90/$ 03.50

EXPERIMENTAL

General methods. — Preparative h.p.l.c. was performed with a JASCO Twincle pump and a VL-611 variable-loop injector with a Showa Denko SE-11 refractive index (r.i.) monitor. H.p.l.c. analyses were performed either with a JASCO 880-PU pump, a Waters U6K universal injector, and a Showa Denko SE-61 r.i. monitor, or with a Dionex BioLC Model 4000i system equipped with a PAD 2 pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode and a silver–silver chloride reference electrode and a potentiostat. The columns used were a YMC-Pack A-312 (150 × 6 mm i.d.) (Yamamura Chemical), a Hibar LiChrosorb NH_2 (250 × 4 mm i.d.) (Merck), an ERC-NH-1171 (200 × 6 mm i.d.) (Erma Optical Works), a Hypercarb (100 × 4.6 mm i.d.) (Shandon), and an HPIC-AS6 (250 × 4 mm i.d.) (Dionex). For preparative chromatography, a YMC-Pack SH-343-5 AQ column (250 × 20 mm i.d.) (Yamamura Chemical) was used. H.p.l.c. analyses at constant temperature were conducted by the use of a column oven SSC 3510C (Senshu Scientific Co.). A Shimazu Chromatopac C-R1A digital integrator was used for quantitative analysis.

F.a.b.-m.s. was performed with a JEOL JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy equivalent to 6 kV at an accelerating voltage of 3 kV. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark), and glycerol was used as the matrix solution. $(G)_2$-βCDs were analyzed in both the positive- and negative-ion modes.

^{13}C-N.m.r. spectra (at 50.10 MHz and 125.65 MHz) were recorded at ambient temperature on 2–3% solutions in D_2O with JEOL JNM-FX 200 and GSX-500 spectrometers. A micro cell was used, and chemical shifts are expressed in p.p.m. downfield from the signal of Me_4Si using 1,4-dioxane (67.40 p.p.m.) as the external standard. The F.t.-n.m.r. conditions were as follows: spectral width 3000 Hz, pulse flipping angle 45°, number of data points 16 384. The delay time for the insensitive nuclei enhanced by the polarization transfer (INEPT) method[3] was 5.1 ms (3/4J). The conditions for ^{1}H[^{1}H]-shift-correlated 2D n.m.r. spectroscopy (COSY) and ^{13}C[^{1}H]-shift-correlation (C–H COSY) measurements were as follows: spectral width 1100 Hz (6002

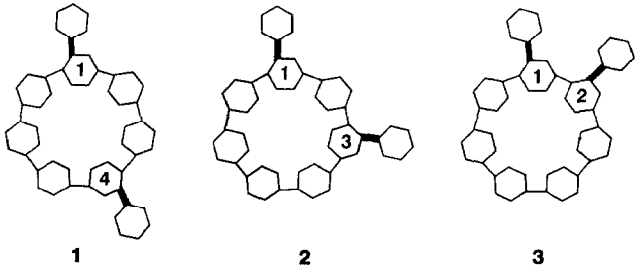

Fig. 1. Positional isomers **1**, **2**, and **3** of diglucosyl-βCD schematically illustrated. ⬡ Glucose unit, — α-(1→4)-linkage, ▬ α-(1→6)-linkage.

Hz), pulse flipping angle 90° (90°), matrix size 2k × 1k (512 × 4k).

Materials. — A mixture of branched βCDs, prepared as previously reported[1], was kindly donated by Sanraku, Ltd. All reagents were of analytical grade. Reagent-grade organic solvents used for chromatography were dried and freshly distilled before use. Water used in solvent preparations was distilled, deionized, and redistilled. The sodium hydroxide solution used as the eluent in chromatography on an HPIC-AS6 column was prepared by dilution of carbonate-free 50% sodium hydroxide solution with deionized water (18 MΩ cm) purified using a NANO-pure II system (Barnstead).

Fragmentation analysis. — A $(G)_2$-βCD sample (3 mg) was partially hydrolyzed in 0.5M trifluoroacetic acid (1 mL) for 150 min at 100°. The solution containing the hydrolyzates was evaporated to dryness under diminished pressure. The residue was dissolved in water (100 μL), and aliquots (2 μL) were analyzed by h.p.l.c.

RESULTS AND DISCUSSION

Isolation. — A mixture of branched βCDs, prepared as previously reported[1], was subjected to preparative h.p.l.c. on a YMC-Pack SH-343-5 AQ ODS column. The loading capacity of this column was about 30 mg for this separation. First, using 13% methanol as the eluent at a flow-rate of 5 mL · min^{-1}, a fraction containing $(G)_2$-βCDs was separated from the slower eluting glucosyl-βCD fraction, and then the $(G)_2$-βCD fraction was rechromatographed using 10% methanol at a flow-rate of 6 mL·min^{-1}. The elution order of three positional isomers was **3** [retention time (t_R) = 19.3 min], **2** (t_R = 20.5 min), and **1** (t_R = 21.2 min), and, as the amount of **3** was very small, making base-line separation of **3** and **2** possible (R_s = 1.07), these two isomers were relatively easily isolated, whereas **1**, which eluted immediately after **2**, was difficult to isolate in pure form by h.p.l.c. Fortunately, **1** was readily crystallized from a mixture of **1** and **2** from water. By a combination of repeated crystallizations and preparative h.p.l.c. runs, the three positional isomers of $(G)_2$-βCD were each isolated in a highly purified state.

The relative abundance of **1**, **2**, and **3**, as measured by h.p.l.c. on a YMC-Pack A-312 column with 3% methanol at a flow-rate of 1 mL·min^{-1} was 74.9:23.3:1.8.

Characterization. — It was confirmed by f.a.b.-m.s. in both positive- and negative-modes that these three compounds (**1**, **2**, and **3**) have the same molecular weight, 1458 daltons.

In a previous paper[1] we reported that three series of linear saccharides were found in the chromatogram of partial hydrolyzates of **1**, as obtained by h.p.l.c. on an amino-bonded silica column. One of three was a series of malto-oligosaccharides (d.p. 2–7). The second series consisted of malto-oligosaccharides each containing one, 1→6 linkage (d.p. 2–8), while the third series, each of which contained two, 1→6 linkages, consisted of four members, the smallest member of which had d.p. 6. This fact indicates that the structure of **1** is 6^1,6^4-di-*O*-α-(D-glucopyranosyl)cyclomaltoheptaose. Similarly, h.p.l.c. analyses of partial hydrolyzates of newly isolated **2** showed the presence of three series of linear saccharides, the first and second of which were the same as those found in

the chromatogram of the partial hydrolyzates of **1**. The third series consisted of five members, the smallest of which had d.p. 5. Therefore, **2** is $6^1,6^3$-di-*O*-(*α*-D-glucopyranosyl)cyclomaltoheptaose (see Fig. 1). Consequently, the remaining one, **3** is thought to be $6^1,6^2$-di-*O*-(*α*-D-glucopyranosyl)cyclomaltoheptaose, because one of the other isomers, having the same molecular weight, 6-*O*-*α*-isomaltosyl-cyclomaltoheptaose cannot be formed by this preparation from potato starch with pure CGTase, and another one, 6-*O*-(*α*-maltosyl)cyclomaltoheptaose can be clearly distinguished from **3** by its ^{13}C-n.m.r. spectrum and chromatographic behavior. There is a linear relationship between the d.p. of a series of malto-oligosaccharides and their t_R, as shown by a plot of log t_R *vs.* d.p. which gave a straight line (Fig. 2).

^{13}C-N.m.r. spectroscopy (Fig. 3) of the three positional isomers **1**, **2**, and **3** offered further corroborating evidence relating to their structures. The ^{13}C-resonances of ring carbons in the spectrum of **1** were assigned using the 2D method. The spectra of **2** and **3** are similar to that of **1**, and hence assignments of signals could be made by analogy (see Table I). In the spectra of **1**, **2**, and **3**, two types of C-1 signals were observed, one of which is the CD ring C-1, and the other of which is the side-chain C-1 signal. The side-chain C-1 signal of **1** and **2** appeared as a single peak, whereas that of **3** was split into two peaks. In common with the three compounds, coupling constants ($J_{C,H}$) of both C-1 atoms are 170 Hz, and, therefore, the configuration of C-1 in the branched residue is *α*, as well as that of C-1 in the CD ring. The integrated ratios in the intensities of the ring C-1 signal and the side-chain C-1 signal are 7:2 for all three. The assignments of three kinds of C-6 signals were confirmed by the INEPT mehtod, using $\Delta = 3/4J$. A large downfield shift of one C-6 signal ($\sim$68 p.p.m.) indicates that the side-chain D-glucose residue is attached to oxygen on this carbon atom[4]. The ratio in the signal intensities of the ring C-6, the side-chain C-6, and the branch-point C-6 was 5:2:2. The branch-point

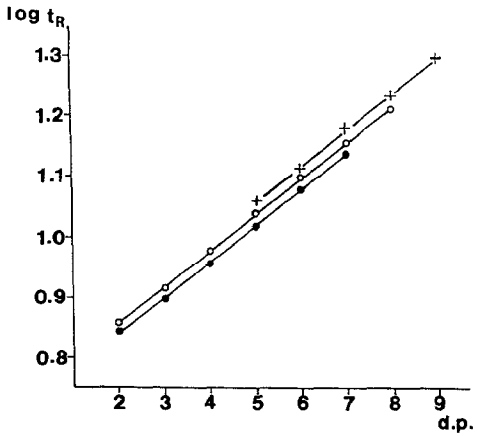

Fig. 2. Relationship between log t_R and d.p. ●—●, purely *α*-(1→4)-linked D-*gluco*-oligomers; ○—○, *α*-(1→4)-linked D-*gluco*-oligomers, each containing one, 1→6 linkage; +—+, *α*-(1→4)-linked D-*gluco*-oligomers, each containing two, 1→6 linkages. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm i.d.); eluent, 63% acetonitrile; flow rate, 1 mL·min^{-1}; temperature, ambient.

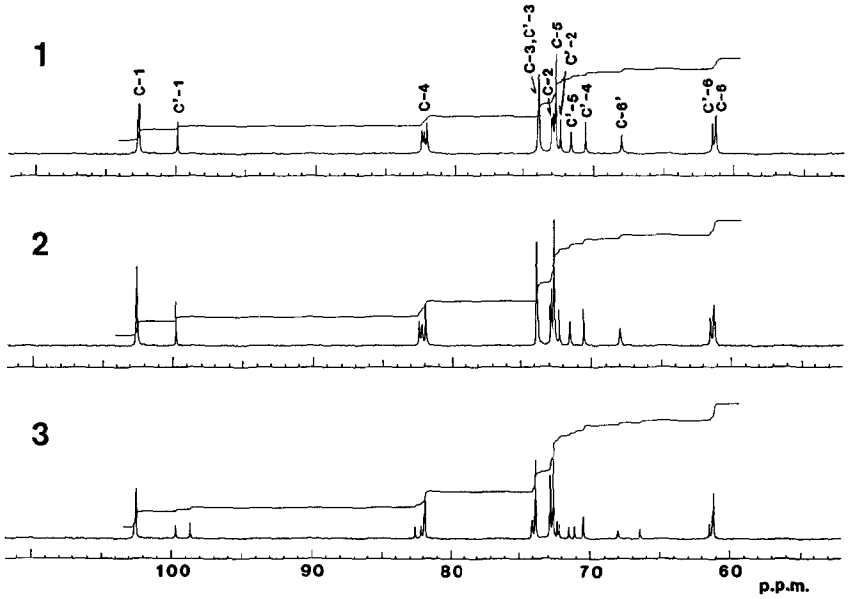

Fig. 3. ^{13}C-N.m.r. spectra of the isomeric diglucosyl-βCDs **1**, **2**, and **3** measured in D_2O at 50.10 MHz. C: the carbon atom of the ring D-glucose unit. C': the carbon atom of the branched unit. C-6': the carbon atom of the branch point.

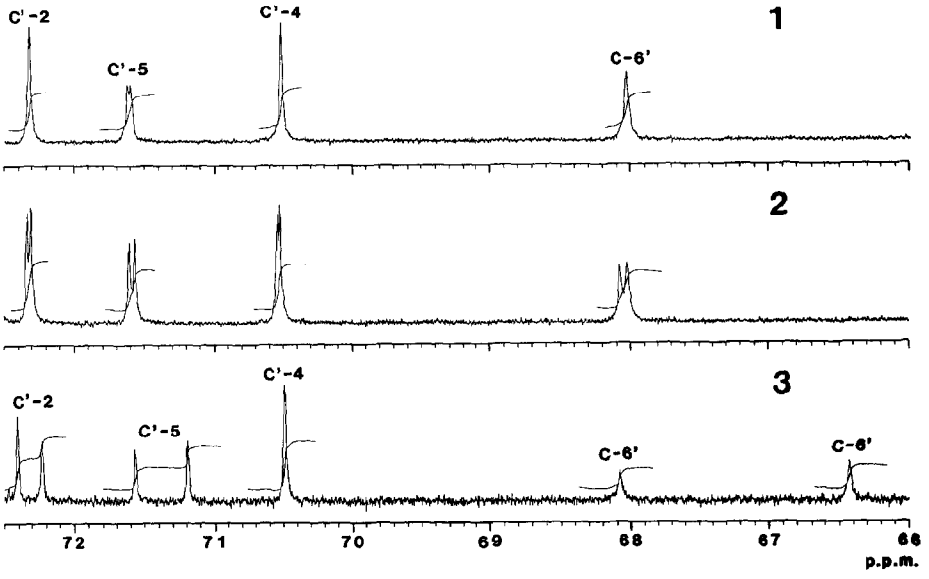

Fig. 4. Expanded ^{13}C-n.m.r. spectra of the 66–72.5 p.p.m. region of **1**, **2**, and **3** at 125.65 MHz.

TABLE I

^{13}C-chemical shifts (125.65 MHz, in D_2O) for three positional isomers of diglucosylcyclomaltoheptaose (**1**, **2**, and **3**)[a]

Atom	**1**		**2**		**3**	
	δ(p.p.m.)	*Integral*	*δ(p.p.m.)*	*Integral*	*δ(p.p.m.)*	*Integral*
C-1	102.62	7	102.63	7	102.62	7
	102.64		102.65		102.67	
	102.66		102.67		102.75	
	102.68		102.75			
	102.72					
C'-1	99.88	2	99.86	2	98.73	2
					99.80	
C-2	72.90	7	72.91	7	72.93	7
	72.93		72.93		72.94	
	73.00		72.98		73.00	
			72.99			
C'-2	72.32	2	72.30	2	72.24	2
			72.33		72.41	
C-3 and	73.83	9	73.82	9	73.87	9
C'-3	73.87		73.83		73.93	
	73.92		73.85		74.09	
	73.95		73.88		74.18	
			73.92			
			73.96			
C-4	82.03	5	82.01	5	81.94	5
	82.08		82.04		81.99	
	82.27		82.26		82.01	
	82.30		82.33		82.05	
C-4'	82.42	2	82.47	2	82.26	2
	82.44		82.51		82.66	
C'-4	70.52	2	70.52	2	70.50	2
			70.54			
C-5	72.69	7	72.68	7	72.68	7
	72.74		72.75		72.71	
	72.76		72.77		72.75	
					72.79	
C'-5	71.61	2	71.56	2	71.19	2
	71.63		71.61		71.57	
C-6	61.26	5	61.24	5	61.22	5
	61.28		61.30		61.23	
	61.29		61.32		61.24	
	61.30					
	61.31					
C-6'	68.03	2	68.02	2	66.42	2
			68.07		68.08	
C'-6	61.48	2	61.47	2	61.29	2
			61.51		61.48	

[a] C is the carbon atom of the ring D-glucopyranose unit. C-4' and C-6' are the carbon atoms of the ring D-glucopyranose unit involved in branching. C' is the carbon atom of the side chain D-glucopyranose unit.

C-6 signal of **3** was also split into two peaks. In the expanded spectra of the 66–72.5 p.p.m. region as measured with a GSX-500 spectrometer, the branch-point C-6 signal of **1** appeared as a single peak, whereas that of **2** was slightly split into two peaks (Fig. 4). The same pattern was observed at the side-chain C-2 and C-5 signals. These facts indicate that the largest interaction between two side-chain D-glucose units is in the molecule of **3**, whereas the D-glucose units of **1** are little affected by each other. Thus ^{13}C-n.m.r. spectroscopy substantiated the original assignments of the structures of three positional isomers. That is, **1**, **2**, and **3** are the $6^1,6^4$-, $6^1,6^3$-, and $6^1,6^2$-substituted isomers, respectively. Interestingly, these three compounds can be clearly distinguished from maltosyl-βCD in terms of the C-4 resonances. In the ^{13}C-n.m.r. spectrum of maltosyl-βCD[5], three types of C-4 signals were observed: ring C-4 (R) at $\sim$82 p.p.m., side-chain C-4 bonded to the other side-chain residue (S) at 78.9 p.p.m., and C-4 of the side-chain residue with free OH (So) at 70.4 p.p.m. The ratio in the signal intensities of R, S, and So was 7:1:1. On the other hand, each diglucosyl-βCD showed only two kinds of C-4 signal (R and So), and the relative intensities of both signals were 7:2.

The aqueous solutions of three positional isomers of $(G)_2$-βCD, purified by h.p.l.c. and lyophilized, showed almost identical $[\alpha]_D^{30}$ values; +165.5° for **1**, +165.8° for **2**, and +166.1° for **3**. Of the three isomers, only **1** crystallized readily from water and gave m.p. 289–290° (dec.).

Chromatographic behavior. — The three positional isomers showed characteristic chromatographic behavior on four h.p.l.c. columns of different separation modes. The movement of $6^1,6^2$-disubstituted isomer **3** was especially distinct from those of other two isomers.

Figs. 5–8 show the elution profiles of three $(G)_2$-βCDs (**1**, **2**, and **3**) on YMC-Pack A-312 (Fig. 5), on Hibar LiChrosorb NH_2 (Fig. 6); on Hypercarb (Fig. 7), and on HPIC-AS6 (Fig. 8).

YMC-Pack A-312 is a reversed-phase column. Thus the separation mechanism is probably an example of hydrophobic chromatography, namely, increased retention with decreasing solubility in water[6]. The isomer **1** having the longest t_R value on this column is the most easily crystallized from water.

The elution sequence with the NH_2-bonded silica and an acetonitrile–water system follows the order of molecular size[7]. Therefore, three $(G)_2$-βCDs having the same molecular size are difficult to separate from each other on Hibar LiChrosorb NH_2. However, this column assists in determining the approximate molecular weight of each homologue.

Hypercarb is a graphitized carbon column, and the retention characteristics of compounds on the column are the result of an adsorption mechanism. Thus far this column has not been used for separation of CDs. The unique resolving power of this column led to an excellent separation of all three isomers. Moreover, a trace contamination of **2** in **1**, which had escaped detection by h.p.l.c. on the ODS column, was definitively determined.

HPIC-AS6 is a strongly basic anion-exchange column in the hydroxide form, and carbohydrates can be separated by anion-exchange with highly alkaline eluents, as

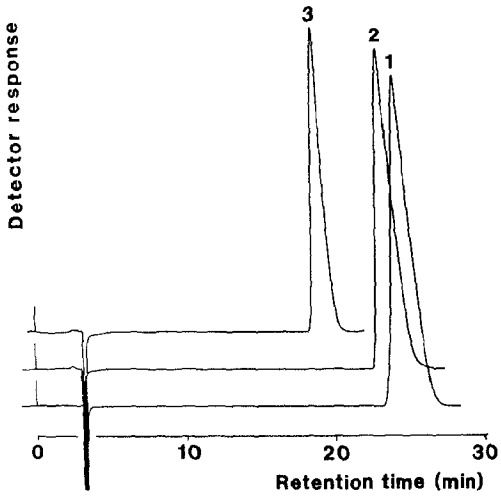

Fig. 5. Elution profiles of compounds **1**, **2**, and **3** on YMC-Pack A-312 (150 × 6 mm i.d.) with 4% methanol. Flow rate: 1 mL·min^{-1} at 35°. Detector: SE-61 r.i.

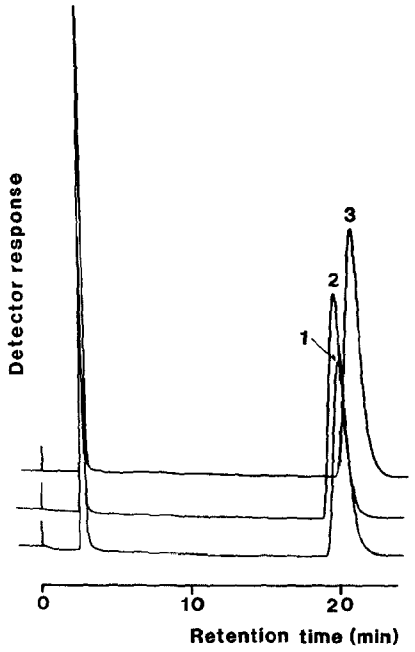

Fig. 6. Elution profiles of compounds **1**, **2**, and **3** on Hibar LiChrosorb NH_2 (250 × 4 mm i.d.) with 67% acetonitrile. Flow rate: 1 mL·min^{-1} at 35°. Detector: SE-61 r.i.

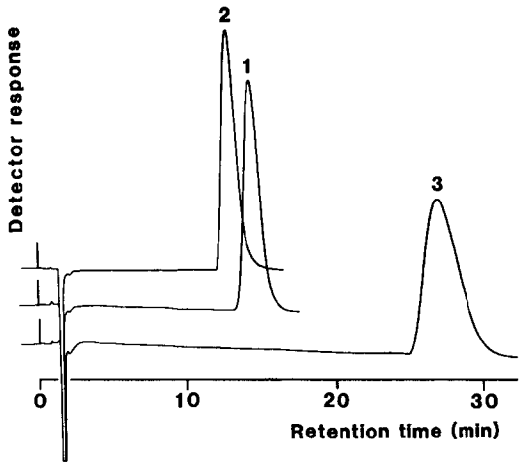

Fig. 7. Elution profiles of compounds 1, 2 and 3 on Hypercarb (100 × 4.6 mm i.d.) with 10% acetonitrile. Flow rate: 1 mL · min^{-1} at 35°. Detector: SE-61 r.i.

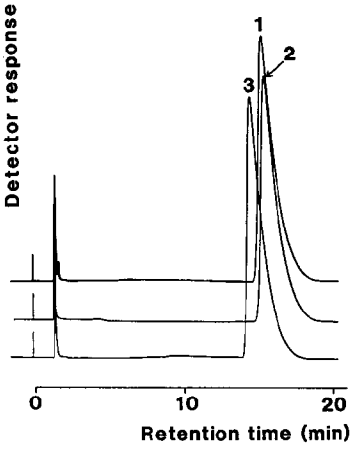

Fig. 8. Elution profiles of compounds 1, 2 and 3 on HPIC-AS6 (250 × 4 mm i.d.) with 150mM sodium hydroxide–150mM sodium acetate. Flow rate: 1 mL · min^{-1} at 25°. Detector: PAD 2.

carbohydrates have pK_a values[8] ranging from pK_a 12–14. However, the ion-exchange resin used for the column consists of a 10-μm particle which is composed of a surface sulphonated PS/DVB core and a monolayer of anion-exchange latex. The ion-exchange capacity is less than 1% of that of ordinary anion-exchange resin. The elution order of CDs was cyclomalto-hexaose (αCD), -octaose (γCD), and -heptaose (βCD)[9]. This result indicates that the elution mechanism on this column is not only by simple anion-exchange, but also involves some hydrophobic interactions. The elution profiles of **1** and **2** were quite similar to those on an NH_2-bonded silica column, whereas **3** eluted before **1** and **2** as on the ODS column. Although this h.p.l.c. system is unsuitable for

separation of the positional isomers, the detector sensitivity is superior to that of an r.i. detector. Thus the sample size required for this analysis was less than one-tenth that required by the other systems mentioned above.

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