Carbohydrate Research, 192 (1989) 159–166
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ISOLATION AND CHARACTERIZATION OF HEPTAKIS(2,6-DI-*O*-METHYL)CYCLOMALTOHEPTAOSE AND OVER-METHYLATED HOMOLOGUES

YOKO KUBOTA, TOSHIKO TANIMOTO, SIZUYO HORIYAMA, AND KYOKO KOIZUMI*
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya 663 (Japan)
(Received December 24th, 1988; accepted for publication, February 28th, 1989)

ABSTRACT

From a mixture of methylation products of cyclomaltoheptaose (β-cyclodextrin, βCD) containing heptakis(2,6-di-*O*-methyl)-βCD (**1**) and its over-methylated homologues, two major components [**1** and hexakis(2,6-di-*O*-methyl)-(2,3,6-tri-*O*-methyl)-βCD (**2**)] and fourteen minor components were isolated by h.p.l.c. The solubilities and inclusion behaviour of **1** and a commercial DM-βCD (a mixture containing **1** and **2**) were compared. A 3-*O*-methyl group had little effect on the inclusion behaviour of 2,6-di-*O*-methyl-βCD. Fourteen minor over-methylated homologues were characterized.

INTRODUCTION

We have reported[1] that a commercial and two synthetic samples of heptakis-(2,6-di-*O*-methyl)cyclomaltoheptaose (DM-βCD) contained two major and at least four minor components, and have isolated one minor and the two major components by semi-preparative h.p.l.c. on a Nova-Pak C_{18} cartridge eluted with 2-propanol–water (19:81). Determination of the molecular weight by f.a.b.-m.s. and successive hydrolysis, reduction, acetylation, and g.l.c.–m.s. of the resulting acetylated partially methylated derivatives of D-glucitol showed that the two major components were heptakis(2,6-di-*O*-methyl)-βCD (**1**) and hexakis(2,6-di-*O*-methyl)-(2,3,6-tri-*O*-methyl)-βCD (**2**), and that the minor one was pentakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl)-βCD.

On the basis of n.m.r. spectroscopy, Spencer *et al.*[2] concluded that their DM-βCD, prepared according to literature procedures[3–5], and all samples of the commercially available, so-called, pure DM-βCD contained only ~65% of **1**. After benzoylation of methylated βCD, the derivatives of **1** and **2** were separated by chromatography and characterized by high-resolution ^{1}H- and ^{13}C-n.m.r. spectroscopy. Pitha and associates[6] also pointed out, on the basis of plasma desorption-m.s.

*Author for correspondence.

0008-6215/89/$ 03.50

data, that the products of laboratory methylation of CDs and several commercial DM-βCD preparations were mixtures.

Despite these findings, the commercially available DM-βCD has been used extensively for measurements of physicochemical properties and for studies of pharmaceutical applications.

We now report on a highly efficient method of isolating pure **1** and on its solubility and complex-forming ability in comparison with those of a commercial DM-βCD. Some properties of the over-methylated homologue **2** were also investigated. Furthermore, fourteen minor products of over-methylated βCD were isolated and characterized.

EXPERIMENTAL

General methods. — The h.p.l.c. system consisted of a JASCO 880-PU pump, a Waters U6K universal injector, and a Showa Denko SE-61 refractive index monitor. The columns (Yamamura Chemical) used were a YMC-Pack A-312 (150 × 6 mm i.d.) and a YMC-Pack SH-343-5 AQ (250 × 20 mm i.d.). For pre-fractionation of partially methylated βCDs, a Lobar LiChroprep Si 60 (63–125 μm) column (440 × 37 mm i.d.) (Merck) was used. H.p.l.c. at constant temperature was conducted using a column oven SSC 3510C (Senshu Scientific Co.).

G.l.c. was carried out with a Hitachi GC 063 instrument. A glass column (2 m × 3 mm i.d.) filled with 3% of ECNSS-M on Chromosorb W (AW-DMCS, 100–120 mesh) was used isothermally at 180°. A Shimadzu Chromatopac C-R3A digital integrator was used for quantitative analyses.

G.l.c.–m.s. was conducted with an MS-SL05 gas chromatograph directly coupled to the source of a Jeol JMS DX-303 mass spectrometer. Low-resolution e.i.-mass spectra were obtained at an ionizing energy of 20 eV, an ionization current of 300 μA, an accelerating voltage of 3 kV, and an ion-source temperature of 270°.

F.a.b.-m.s. was performed in the positive mode with a Jeol JMS-DX 303 mass spectrometer, using xenon atoms having a kinetic energy equivalent to 6 kV. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark), and glycerol or *m*-nitrobenzyl alcohol was used as the matrix solution.

^{13}C-N.m.r. spectra (50.10 MHz) were recorded at ambient temperature on 2–3% solutions in D_2O with a Jeol JNM-FX 200 F.t.-n.m.r. spectrometer. 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.

Materials. — βCD (cyclomaltoheptaose) was used after recrystallization from water. A commercial DM-βCD was donated by Toshin Chemical. Mixtures of **1** and over-methylated homologues were prepared according to the procedure of Szejtli *et al.*[3], using 1.5-fold more than the original amounts of the reagents.

Reagent-grade organic solvents, used for chromatography and synthesis, were dried and freshly distilled before use. Water used in the solvent preparations was distilled, deionized, and redistilled.

Solubility studies. — Water (1.0 mL) and an excess of the lyophilized sample were shaken in a sealed glass vessel for 1 h at 25°, 40°, or 55° (±0.1°). The solutions were passed through a 0.2-μm membrane filter, and the concentration of the solute was determined by h.p.l.c. on a Waters Nova-Pak C_{18} cartridge (100 × 8 mm i.d.; methanol–water, 13:87 for βCD, and 85:15 for **1** and a commercial DM-βCD); **1** and **2** in the commercial DM-βCD appeared as a single peak.

The inclusion behaviours of **1**, **2**, and a commercial DM-βCD were determined by the solubility method[7] as described[8] for branched CDs.

Fragmentation analysis. — Each methylated βCD (10 mg) was hydrolyzed in 2M trifluoroacetic acid (0.5 mL) overnight at 100°. The solution was concentrated to dryness under reduced pressure, and a solution of $NaBH_4$ (200 mg) in water (2 mL) was stirred with the residue for 3 h at room temperature. Conventional work-up gave partially methylated D-glucitol derivatives that were acetylated with acetic anhydride and pyridine, and solutions of the products in acetone were analysed by g.l.c.–m.s.

RESULTS AND DISCUSSION

H.p.l.c. of heptakis(2,6-di-O-methyl)-βCD (**1**) *and over-methylated homologues.* — First, an attempt was made to characterize the minor unknown

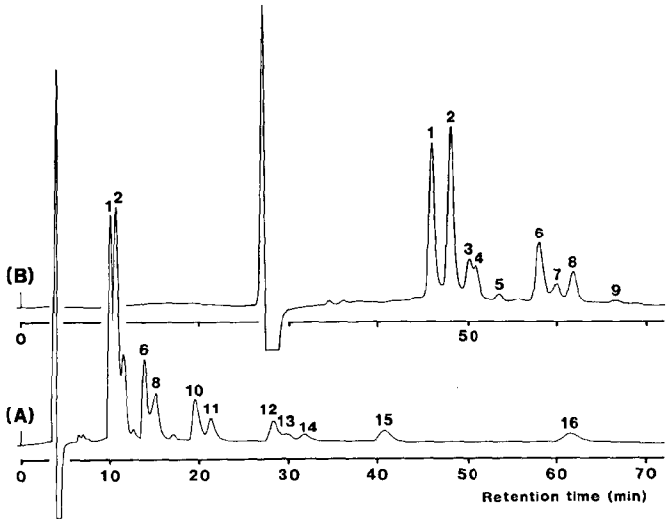

Fig. 1. (A) Elution profile of a mixture of partially methylated βCDs on YMC-Pack A-312 (150 × 6 mm i.d.) with 1-propanol–water (16:84); (B) separation of the fast-moving components on YMC-Pack SH-343-5 AQ (250 × 20 mm i.d.) with 2-propanol–water (25:75). Flow rate, (A) 1 mL/min, (B) 2 mL/min at 35°.

components detected in the previous study[1]. A mixture of partially methylated βCDs containing relatively large amounts of the desired minor components was prepared by modification of the method of Szejtli *et al.*[3] with increased amounts of reagents.

Re-investigation of the chromatographic system led to the choice of YMC-Pack A type ODS columns, which were efficient for the separation of CDs and branched CDs[9]. Fig. 1 shows the elution profiles of a mixture of partially methylated βCDs and the fast-moving components. With βCD as the test sample, the theoretical plate number of the A-312 column was 7,280 and that of the SH-343-5 AQ column was 26,730. Therefore, the latter made possible fine separations of partially methylated βCDs, and base-line separation of the two major components, **1** and **2**, was achieved. Moreover, the capacity of the SH-343-5 AQ column was ~15-fold more than that of the Nova-Pak C_{18} cartridge (100 × 8 mm i.d.) that was used in the previous work[1]. Thus, ~30 mg of sample could be loaded for the isolation of **1**, **2**, and the fourteen over-methylated homologues pre-fractionated on a Lobar LiChroprep Si 60 column with benzene–ethanol. The principal eluent was

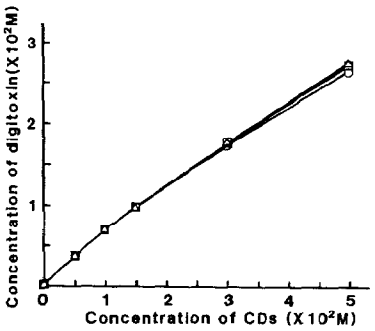

Fig. 2. Phase solubility diagrams of digitoxin–partially methylated βCD systems in water at 25°: **1**, ○; **2**, △; commercial DM-βCD, □.

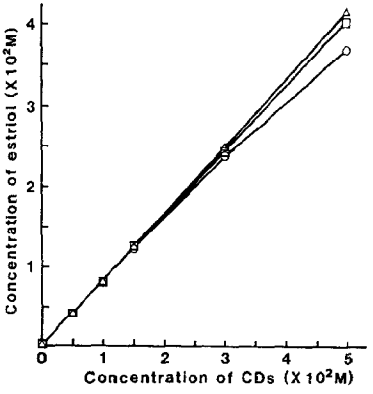

Fig. 3. Phase solubility diagrams of estriol–partially methylated βCD systems in water at 25°. Symbols as in Fig. 2.

17–30% 1-propanol with 20–23% 2-propanol being used for some components that were difficult to separate under these conditions, *e.g.*, peaks 3 and 4.

For the most efficient isolation of **1**, βCD was methylated according to the procedure of Boger *et al.*[5], the products were crystallized from methanol, and the crystalline material was chromatographed as described above. The ratio of **1**:**2** increased on crystallization of the mixture from methanol.

Solubility of heptakis(2,6-di-O-methyl)-βCD (**1**). — The solubilities of pure **1** in water at 25°, 40°, and 55° were measured and compared (Table I) with those of a commercial DM-βCD (containing **1**, **2**, and several minor homologues; see Table II) and the parent βCD. In general, a contaminant having a similar constitution increases the solubility. The solubility of pure **1** is considerably lower than that of the commercial DM-βCD.

The solubility of the over-methylated homologue **2** in water was somewhat higher than that of **1** despite having one less hydroxyl group and, like that of **1**, decreased abruptly with rising temperature. Therefore, recrystallization of a mixture of **1** and **2** by heating an aqueous solution induced co-precipitation, and purification of **1** by recrystallization from water was unsuccessful.

On the other hand, the solubilities of the methylated βCDs in methanol increased normally with rising temperature and **2** was more soluble than **1**. Therefore, fractional crystallization from methanol was possible to a certain extent.

Inclusion behaviour. — The complex-forming abilities of **1**, **2**, and the commercial DM-βCD with digitoxin and estriol in water were studied by the solubility method. The minimum requirement for inclusion complex formation is size compatibility between the host and guest molecules[10]. The influence of a 3-*O*-methyl group had little effect on the inclusion behaviour of 2,6-di-*O*-methyl-βCD.

TABLE I

SOLUBILITIES (g/100 mL) OF DI-*O*-METHYL-βCD AND βCD IN WATER

Compound	*25°*	*40°*	*55°*
Heptakis(2,6-di-*O*-methyl)-βCD	45.03	11.85	2.72
Commercial DM-βCD	65.80	31.27	6.13
βCD	1.95	3.38	5.29

TABLE II

COMPOSITION (%) OF THE METHYLATION PRODUCTS OF βCD (A) AND THE COMMERCIAL DM-βCD (B)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
A	20.7	24.4	6.5	4.8	1.5	9.5	3.2	5.1	0.5	7.1	4.1	4.1	1.4	1.6	3.0	2.5
B	40.3	37.7	3.9	3.4		7.7		1.5		5.5						

Figs. 2 and 3 show the phase solubility diagrams obtained for digitoxin and estriol with **1**, **2**, and the commercial DM-βCD at 25°. The complexation abilities of these compounds seem to be almost the same. Slight differences between the solubilities of estriol in solutions of **1**, **2**, and the commercial DM-βCD of higher concentrations were observed. This phenomenon suggests that the solubility of the estriol–**1** complex is lower than that of the estriol–**2** complex. These results indicate that the commercial DM-βCD is superior to pure **1** as the host compound of inclusion complexes, owing to its higher solubility.

Comparison of physicochemical properties of **1** *and* **2**. — Pure **2** became colored at ~275° and then melted at 280–284°, whereas **1** became colored at 276°[1], but did not melt even at 300°. The $[\alpha]_D$ values were +168.9° for **2** and 162.5° for **1** (*c* 1, water).

Fig. 4. shows ^{13}C-n.m.r. spectra of **1** and **2** in D_2O. The ^{13}C-resonances of the ring carbons in the spectrum of **1**, assigned[11] using the 2D method, were confirmed with a Jeol GSX 500 spectrometer. Suzuki *et al.*[12] elucidated the chemical shifts of the ^{13}C-resonances of MeO-2 and MeO-6, respectively, at 60.1 and 58.6 p.p.m. in **1** and at 58.6 and 58.9 p.p.m. in permethylated βCD. The signal for MeO-3 appeared at lowest field (60.1 p.p.m.). Spencer *et al.*[2] also assigned the chemical shifts of the resonances of the ring and methoxyl carbons of **1** in C_6D_6. From a comparison of the ^{13}C-shift data for βCD, 2,6-di-*O*-methyl-βCD (**1**) and 2,3,6-tri-*O*-methyl-βCD (**17**), the effect of methylation of HO-3 could be deduced.

Methylation of one HO-3 (**1**→**2**) caused an α-shift of ~ +9 p.p.m. for the C-3 resonance and β-shifts of ~ −1.1 and ~ −2.2 p.p.m. for the resonances of C-2 and C-4, respectively. A signal for MeO-3 appeared at 61.5 p.p.m., and one of the MeO-2 signals was shifted to 58.8 p.p.m. as in the spectrum of **17**. However, as these changes were relatively small, the ^{13}C-n.m.r. spectrum (D_2O) of **1** contaminated by **2** may imply that it is a pure compound.

Characterization of fourteen minor over-methylated homologues. — The minor homologues **3**–**16**, corresponding to the peaks 3–16 in Fig. 1, were isolated by h.p.l.c. on YMC-Pack A type ODS columns, as mentioned above. A trace of **17**

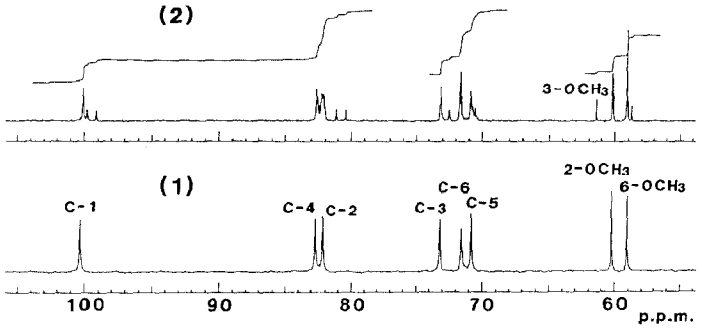

Fig. 4. ^{13}C-N.m.r. spectra of heptakis(2,6-di-*O*-methyl)-βCD (**1**) and hexakis(2,6-di-*O*-methyl)-(2,3,6-tri-*O*-methyl)-βCD (**2**) in D_2O.

TABLE III

EXPECTED FRAGMENT IONS IN F.A.B-M.S. SPECTRA OF THREE POSITIONAL ISOMERS[a]

m/z	*Corresponding to*	**3**	**4**	**6**
191	[● + H]$^+$	○	○	○
205	[○ + H]$^+$	○	○	○
381	[2● + H]$^+$	○	○	○
395	[● + ○ + H]$^+$	○	○	○
409	[2○ + H]$^+$	×	×	○
571	[3● + H]$^+$	○	○	○
585	[2● + ○ + H]$^+$	○	○	○
599	[● + 2○ + H]$^+$	×	○	○
761	[4● + H]$^+$	×	○	○
775	[3● + ○ + H]$^+$	○	○	○
789	[2● + 2○ + H]$^+$	○	○	○
951	[5● + H]$^+$	×	×	○
965	[4● + ○ + H]$^+$	○	○	○
979	[3● + 2○ + H]$^+$	○	○	○
1155	[5● + ○ + H]$^+$	○	○	○
1169	[4● + 2○ + H]$^+$	○	○	○

[a] ● = 2,6-Me_2-Glc, ○ = 2,3,6-Me_3-Glc.

was also detected (*T* 135 min under the conditions of Fig. 1A). The composition (%) of the products of methylation of βCD, prepared following the procedure of Szejtli *et al.*[3] with 1.5-fold more than the original amounts of the reagents, is summarized in Table II together with the composition of the commercial DM-βCD.

Each product was successively hydrolyzed, reduced, and acetylated, and the resulting D-glucitol derivatives were characterized by g.l.c.–m.s. There was only one product from **1**, namely, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-glucitol (2,6-Me_2) as mentioned before[1]. The products from **2**–**16** contained only 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (2,3,6-Me_3) in addition to 2,6-Me_2. The ratios of 2,6-Me_2 and 2,3,6-Me_3 obtained for **3**–**16** and their molecular weights, confirmed by f.a.b.-m.s., indicate that **3**, **4**, and **6** are positional isomers of pentakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl-βCD; **5**, **7**, **8**, and **10** are four of five isomers of tetrakis(2,6-di-*O*-methyl)-tris(2,3,6-tri-*O*-methyl)-βCD; **9**, **11**, and **12** are three of five isomers of tris(2,6-di-*O*-methyl)-tetrakis(2,3,6-tri-*O*-methyl)-βCD; **13**, **14**, and **15** are three isomers of bis(2,6-di-*O*-methyl)-pentakis(2,3,6-tri-*O*-methyl)-βCD; and **16** is (2,6-di-*O*-methyl)-hexakis(2,3,6-tri-*O*-methyl)-βCD. Thus, of the eighteen theoretically possible over-methylated homologues of **1**, one major and fourteen minor homologues were isolated and characterized.

The separation on ODS, which is probably hydrophobic chromatography, did not follow the d.s., since the following orders of elution of **5** (d.s. 15) and **6** (d.s. 14) and of **9** (d.s. 18) and **10** (d.s. 17) were reversed.

The structures of three positional isomers (**3**, **4**, and **6**) of pentakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl)-βCD were investigated by f.a.b.-m.s. Table III shows the fragment ions produced by cleavage of glucosidic bonds. It was expected that the ions at *m/z* 409, 599, 761, and 951 would be characteristic. With glycerol

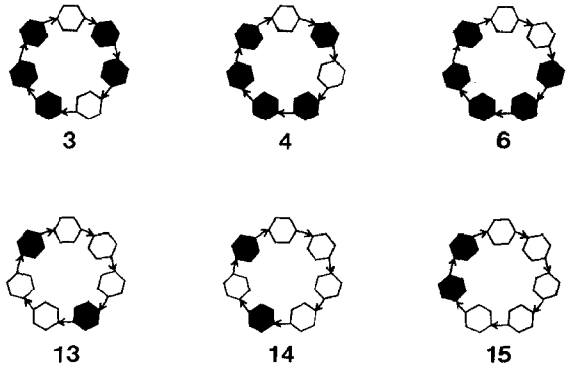

Fig. 5. Positional isomers of pentakis(2,6-di-*O*-methyl)-bis(2,3,6-tri-*O*-methyl)-βCDs (**3**, **4**, and **6**) and bis(2,6-di-*O*-methyl)-pentakis(2,3,6-tri-*O*-methyl)-βCDs (**13**, **14**, and **15**): ●, 2,6-di-*O*-methylglucose residue (mol. wt. 190); ○, 2,3,6-tri-*O*-methylglucose residue (mol. wt. 204). The numbers correspond with the peaks in Fig. 1.

as the matrix, the fragment at *m/z* 409 was observed only in the spectrum of **6**. With *m*-nitrobenzyl alcohol as the matrix, the fragment at *m/z* 599 was not observed, whereas the fragments at *m/z* 761 and 951 were present in each spectrum. However, the relative intensities of the peaks at *m/z* 761 and 951 compared, respectively, with those of peaks at *m/z* 775 and 965 in each spectrum were different, namely, the relative intensities of *m/z* 761 and 775 were 0.56, 1.00, and 2.55 in the spectra of **3**, **4**, and **6**, respectively, and those of *m/z* 951 and 965 were 0.36, 0.57, and 1.00, respectively. Although these studies did not provide conclusive proof of the structure, because of additional unknown fragmentation, a comparison of the characteristic fragments in f.a.b.-m.s. spectra of **3**, **4**, and **6** suggested that the structures were as shown in Fig. 5. Similar study of the structures of three isomers of bis(2,6-di-*O*-methyl)-pentakis(2,3,6-tri-*O*-methyl)-βCD (**13–15**) suggested the structures shown in Fig. 5. More accurate analysis is in progress.

REFERENCES

1 K. KOIZUMI, Y. KUBOTA, T. UTAMURA, AND S. HORIYAMA, *J. Chromatogr.*, 368 (1986) 329–337.
2 C. M. SPENCER, J. F. STODDART, AND R. ZARZYCKI, *J. Chem. Soc., Perkin Trans. 2*, (1987) 1323–1336.
3 J. SZEJTLI, A. LIPTÁK, I. JODÁI, P. FÜGEDI, P. NÁNÁSI, AND A. NESZMÉLYI, *Staerke*, 32 (1980) 165–169.
4 B. CASU, M. REGGIANI, G. G. GALLO, AND A. VIGEVANI, *Tetrahedron*, 24 (1968) 803–821.
5 J. BOGER, R. J. CORCORAN, AND J.-M. LEHN, *Helv. Chim. Acta*, 61 (1978) 2190–2218.
6 T. IRIE, K. FUKUNAGA, J. PITHA, K. UEKAMA, H. M. FALES, AND E. SOKOLOSKI, *Carbohydr. Res.*, 192 (1989) 167–172.
7 T. HUGUCHI AND K. A. CONNORS, *Adv. Anal. Chem. Instr.*, 4 (1965) 117–212.
8 K. KOIZUMI, Y. OKADA, Y. KUBOTA, AND T. UTAMURA, *Chem. Pharm. Bull.*, 35 (1987) 3413–3418.
9 K. KOIZUMI, Y. KUBOTA, Y. OKADA, T. UTAMURA, S. HIZUKURI, AND J. ABE, *J. Chromatogr.*, 437 (1988) 47–57.
10 F. CRAMER AND H. HETTLER, *Naturwissenschaften*, 54 (1967) 625–632.
11 Y. YAMAMOTO, M. ONDA, Y. TAKAHASHI, Y. INOUE, AND R. CHÛJÔ, *Carbohydr. Res.*, 170 (1987) 229–234.
12 M. SUZUKI, Y. SASAKI, J. SZEJTLI, AND É. FENYVESI, *J. Incl. Phenom.*, 5 (1987) 459–468.