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# ANALYSIS OF HEPTAKIS(2,6-DI-O-METHYL)-β-CYCLODEXTRIN BY THIN-LAYER, HIGH-PERFORMANCE LIQUID AND GAS CHROMATOGRAPHY AND MASS SPECTROMETRY*

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## SUMMARY

Methods for the analysis and isolation of heptakis(2,6-di-O-methyl)-β-cyclodextrin (CD) by thin-layer and high-performance liquid chromatography (HPLC) were investigated. By using these methods a commercial and two synthetic samples of heptakis(2,6-di-O-methyl)-β-CD were analysed and it was found that they contained two major and at least four minor components, of which the two major and one minor components were isolated by semi-preparative HPLC. Careful fragmentation analyses, which consisted of successive hydrolysis, reduction, acetylation and characterization of the partially methylated D-glucitol peracetates by gas chromatography–mass spectrometry and determination of molecular weights by fast-atom bombardment mass spectrometry indicated that these compounds were heptakis(2,6-di-O-methyl)-β-CD, hexakis(2,6-di-O-methyl)-mono(2,3,6-tri-O-methyl)-β-CD and pentakis(2,6-di-O-methyl)-bis(2,3,6-tri-O-methyl)-β-CD.

## INTRODUCTION

Methylated cyclodextrins (CDs) have become of interest in recent years as they have very high solubility in water and also in oil, and their inclusion behaviours are significantly different from those of the parent CDs[1]. Among them, heptakis(2,6-di-O-methyl)-β-CD is expected to be the most useful host molecule for inclusion complexes. This compound was prepared by Boger *et al.*[2] and Szejtli *et al.*[3] from the parent β-CD by treatment with dimethyl sulphate and barium oxide in dimethylformamide–dimethyl sulphoxide (DMF–DMSO) (1:1), followed by recrystallization, and the compound prepared according to the Szejtli *et al.*'s procedure[3] is now commercially available as dimethyl-β-CD (Toshin Chemical, Tokyo, Japan). In addition, Takahashi and Hattori[4] reported an improvement of the methylation of β-CD in which β-CD was treated with dimethyl sulphate and sodium hydroxide in

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two phases consisting of dichloroethane and water. In these preparations, thin-layer chromatography (TLC) on silica gel plates and high-performance liquid chromatography (HPLC) on $C_{18}$-bonded silica with acetonitrile–water were used as tools for the control of the purity of heptakis(2,6-di-O-methyl)-β-CD, and it was reported that each compound obtained gave one spot on TLC and a single peak on HPLC. However, the purity of the compound is doubtful, because it was pointed out[5] that the achievement of selective and efficient modification, especially polysubstitution of CD, is complicated by the statistical problems imposed by the large number of hydroxy groups (21 for β-CD) and, further, the geometry of the molecule is such that for selective polysubstitution, developing steric interactions may tend to reverse the initial selectivity.

This work was undertaken in order to examine the purity of a commercial and two synthetic samples of heptakis(2,6-di-O-methyl)-β-CD by TLC, HPLC, gas chromatography (GC) and fast-atom bombardment mass spectrometry (FAB-MS).

## EXPERIMENTAL

### *General methods*

Melting points were measured with a micro melting point apparatus (Yanagimoto, Kyoto, Japan) and are uncorrected. Optical rotations were determined with a DIP 360 digital polarimeter (Jasco, Tokyo, Japan).

TLC was performed on silica gel 60 TLC plates and silica gel 60 $F_{254}$ HPTLC plates (both from Merck, Darmstadt, F.R.G.) with benzene–acetone–methanol (7:4:1). Methylated β-CD derivatives were detected by spraying with 5% (V/V) concentrated sulphuric acid in ethanol and heating at 100°C.

HPLC analyses were conducted with a Familic-300S pump (Jasco), a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and an RID-98A refractive index monitor (Knauer, Bad Homburg, F.R.G.). The columns used were an ERC-ODS-1171 (200 × 6 mm I.D.) (Erma Optical Works, Tokyo, Japan), a Hibar LiChrosorb RP-18 (250 × 4 mm I.D.) (Merck), a Nova-Pak $C_{18}$ cartridge (100 × 8 mm I.D.) employed in a Waters Z-Module radial compression separation system (Waters Assoc., Milford, MA, U.S.A.) and a YMC-Pack AL-312 ODS (150 × 6 mm I.D.) (Yamamura Chemical, Kyoto, Japan).

GC examinations were carried out using a GC 063 instrument (Hitachi, Tokyo, Japan). A glass column (2 m × 3 mm I.D.) filled with 3% ECNSS-M on Chromosorb W AW-DMCS (100–200 mesh) was used isothermally at 180°C. Other phases for GC, 0.3% OV-275–0.4% GE XF-1150 on Shimalite AW-DMCS (80–100 mesh) and 3% SE-30 on Chromosorb W AW-DMCS (80–100 mesh), were also tested. A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used for quantitative analyses.

GC–MS was conducted with an MS-SL05 gas chromatograph directly coupled to the source of a JMS DX-303 mass spectrometer (Jeol, Tokyo, Japan). Low-resolution EI 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°C.

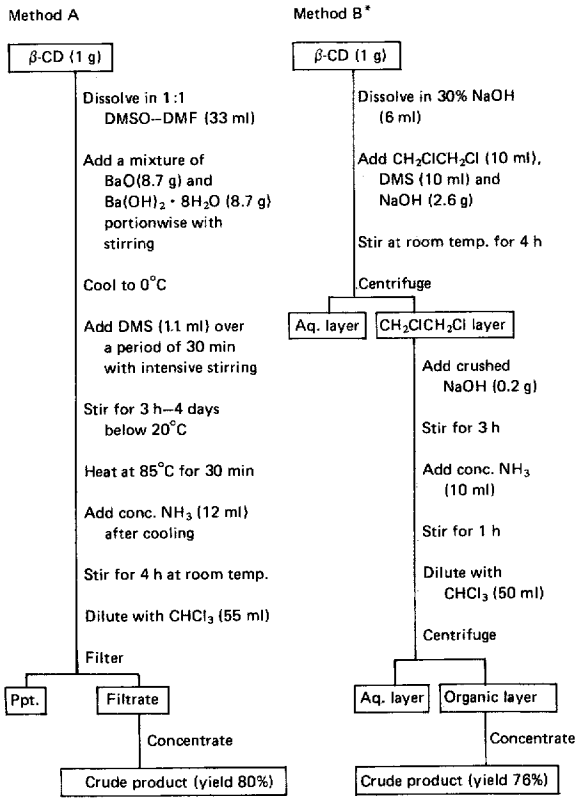
FAB-MS spectrograms of partially methylated β-CD derivatives were obtained on a JMS-DX 303 mass spectrometer using xenon atoms having a kinetic energy

equivalent to 6 kV and in the positive-ion mode. The mass marker was calibrated with perfluoroalkylphosphazine (Ultra Mark) and glycerol was used as the matrix solution.

*Materials*

β-CD, obtained from Hayashibara Biochemical Lab. (Okayama, Japan) was used after recrystallization from water, $[\alpha]_D^{18}$ +165.5° ($c$ = 1, $H_2O$). Methylated β-CD derivatives were donated by Toshin Chemical and were also synthesized in our laboratory according to Szejtli *et al.*'s procedure[3] (method A) and Takahashi and Hattori's procedure[4] (method B) as shown in Fig. 1. All reagents were of analytical-reagent grade.

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.




*Takahashi and Hattori's procedure was partly modified.

Fig. 1. Scheme of two methods for the preparation of heptakis(2,6-di-O-methyl)-β-CD.

*Fragmentation analysis*

A methylated β-CD sample (10 mg) was hydrolysed in 0.5 ml of 2 *M* trifluoroacetic acid overnight at 100°C. The solution containing the hydrolysate was evaporated to dryness under reduced pressure. To the residue were added 200 mg of $NaBH_4$ in 2 ml of water and the mixture was stirred for 3 h at room temperature. After removing $Na^+$ and $H_3BO_3$ and drying, partially methylated D-glucitol derivatives were acetylated with acetic anhydride and pyridine, then the products were dissolved in acetone and analysed by GC–MS.

*Isolation of partially methylated β-CD derivatives*

A commercial sample of dimethyl-β-CD was pre-fractionated by preparative liquid chromatography on a Lobar LiChroprep Si 60 (63–125 μm) column (440 × 37 mm I.D.) (Merck), eluting with benzene–ethanol (20:1), and each fraction was purified by semi-preparative HPLC on a Nova-Pak $C_{18}$ cartridge eluted with 2-propanol–water (19:81).

## RESULTS AND DISCUSSION

*Thin-layer chromatography*

Commercial dimethyl-β-CD showed one spot on silica gel 60 plates (20 cm) developed with benzene–ethanol (4:1)[2], benzene–methanol (4:1)[3] or chloroform–methanol (9:1)[4], but on an HPTLC plate (10 cm) it showed two clearly distinguishable spots on development with benzene–acetone–methanol (7:4:1) (Fig. 2). Duplicate developments with this solvent system permitted the separation of the two spots even on an ordinary silica gel 60 plate (20 cm). The pure sample of heptakis(2,6-di-O-methyl)-β-CD used as a standard was obtained by semi-preparative HPLC as described under Experimental.

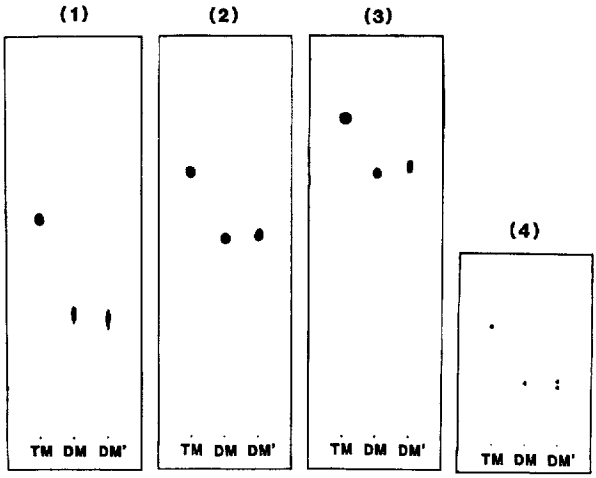



Fig. 2. Thin-layer chromatograms of methylated β-CD derivatives. TM = heptakis(2,3,6-tri-O-methyl)-β-CD; DM = heptakis(2,6-di-O-methyl)-β-CD; DM′ = commercial dimethyl-β-CD. Plates (1), (2) and (3), silica gel 60 TLC (20 cm); (4), silica gel 60 $F_{254}$ HPTLC (10 cm). Solvent: (1) $C_6H_6$–$C_2H_5OH$ (4:1); (2) $C_6H_6$–$CH_3OH$ (4:1); (3) $CHCl_3$–$CH_3OH$ (9:1); (4) $C_6H_6$–$(CH_3)_2CO$–$CH_3OH$ (7:4:1).

The product of synthesis by method A (Fig. 1) gave two major and several minor spots. Repeated recrystallization from water decreased the number of spots to two, which coincided in position with those of the two main components of commercial dimethyl-β-CD. On the other hand, the product of synthesis by method B showed a very complicated chromatogram (Fig. 3). Separation of these overlapping spots was improved by duplicate or triplicate developments, but the isolation of heptakis(2,6-di-O-methyl)-β-CD from this product seemed to be impossible.

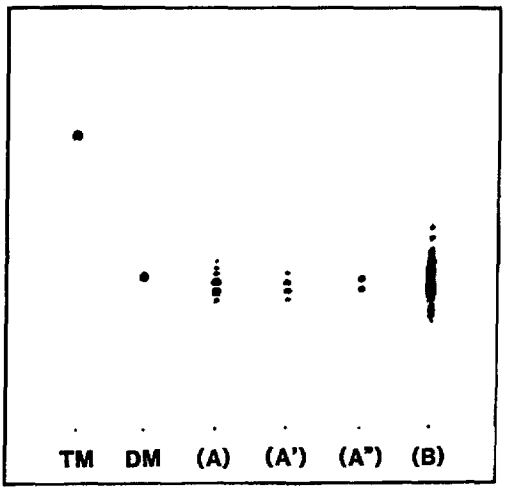



Fig. 3. Thin-layer chromatograms of methylation products of β-CD prepared by methods A and B on a silica gel 60 $F_{254}$ HPTLC plate (10 cm) developed with $C_6H_6$–$(CH_3)_2CO$–$CH_3OH$ (7:4:1). (A) Crude product by method A; (A') recrystallized product from (A); (A") recrystallized product from (A'); (B) crude product by method B. Other details as in Fig. 2.

*High-performance liquid chromatography*

To separate the two main components of commercial dimethyl-β-CD by HPLC, four $C_{18}$-bonded silica columns and three eluent systems (acetonitrile–water, methanol–water and 2-propanol–water) were examined. The following combinations were found to be adequate for this purpose: YMC-Pack AL-312 ODS column eluted with acetonitrile–water (60:40) at 1 ml/min (retention time, $t_R$, of the two main components 6.90 and 7.46 min), ERC-ODS-1171 column eluted with acetonitril–water (60:40) at 0.7 ml/min ($t_R$ 7.91 and 8.05 min) and Nova-Pak $C_{18}$ cartridge column eluted with 2-propanol–water (19:81) at 0.5 ml/min ($t_R$ 22.04 and 23.62 min). Of these, the last combination gave the best separation and the best baseline stability.

Fig. 4 shows the HPLC elution profiles of the methylation products from β-CD obtained by methods A and B. The product of synthesis by method A contained two major and at least four minor components after duplicate recrystallizations, and the composition was almost the same as that of commercial dimethyl-β-CD. The product of synthesis by method B, which could not be recrystallized from water, consisted of many components.

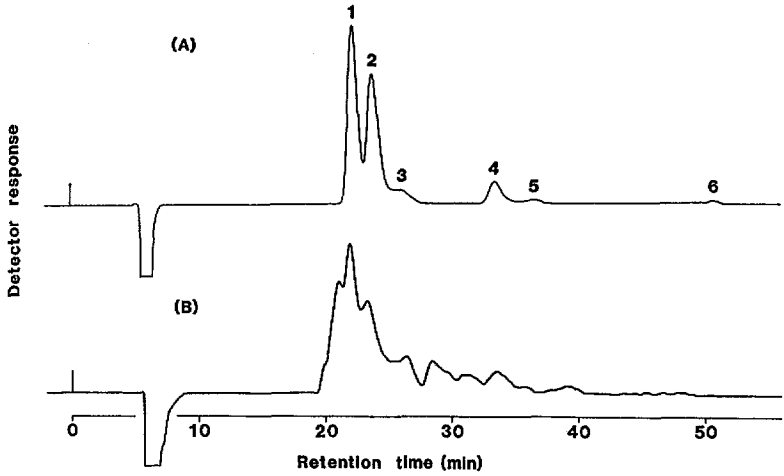



Fig. 4. High-performance liquid chromatograms of methylation products of β-CD prepared by methods A and B. 1 = Heptakis(2,6-di-O-methyl)-β-CD (I); 2 = hexakis(2,6-di-O-methyl)-mono(2,3,6-tri-O-methyl)-β-CD (II); 4 = pentakis(2,6-di-O-methyl)-bis(2,3,6-tri-O-methyl)-β-CD (III); 3, 5 and 6 have not yet been characterized but 3 and 5 are considered to be positional isomers of 4, and 6 may be tetrakis(2,6-di-O-methyl)-tris(2,3,6-tri-O-methyl)-β-CD. Chromatographic conditions: column, Nova-Pak $C_{18}$ cartridge (100 × 8 mm I.D.); eluent, 2-propanol–water (19:81); flow-rate, 0.5 ml/min; temperature, ambient.

*Gas–liquid chromatography and mass spectrometry*

For the fragmentation analysis of methylated β-CD derivatives, separation of the expected eight D-glucitol derivatives (Table I) by GC was attempted. Three phases for GC, 0.3% OV-275–0.4% GE XF-1150, 3% SE-30 and 3% ECNSS-M, were tested. The reproducibility of the retention time ($t_R$) on the first phase was poor. The $t_R$ value on 3% SE-30 at 170°C was the shortest, only 30 min being required for elution of the last peak, but the resolution of the faster eluting peaks was poor. GC on 3% ECNSS-M gave the best result. The relative $t_R$ values of partially methylated D-glucitol acetates and D-glucitol hexaacetate are given in Table I.

TABLE I

RELATIVE RETENTION TIMES OF PARTIALLY METHYLATED D-GLUCITOL PERACETATES AND D-GLUCITOL HEXAACETATE

Chromatographic conditions: column, 3% ECNSS-M on Chromosorb W AW-DMCS (2 m × 3 mm I.D.); column temperature, 180°C; injection temperature, 200°C; carrier gas, nitrogen; flow-rate, 30 ml/min.

| *Compound* | *Abbreviation* | *Relative* $t_R$ |
|---|---|---|
| 1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol | $2,3,6\text{-}OCH_3$ | 0.67 |
| 1,3,4,5-Tetra-O-acetyl-2,6-di-O-methyl-D-glucitol | $2,6\text{-}OCH_3$ | 1.00 |
| 1,2,4,5-Tetra-O-acetyl-3,6-di-O-methyl-D-glucitol | $3,6\text{-}OCH_3$ | 1.11 |
| 1,4,5,6-Tetra-O-acetyl-2,3-di-O-methyl-D-glucitol | $2,3\text{-}OCH_3$ | 1.41 |
| 1,2,3,4,5-Penta-O-acetyl-6-O-methyl-D-glucitol | $6\text{-}OCH_3$ | 1.52 |
| 1,3,4,5,6-Penta-O-acetyl-2-O-methyl-D-glucitol | $2\text{-}OCH_3$ | 2.12 |
| 1,2,4,5,6-Penta-O-acetyl-3-O-methyl-D-glucitol | $3\text{-}OCH_3$ | 2.47 |
| D-Glucitol hexaacetate | Hexaacetate | 3.38 |

TABLE II

PRIMARY FRAGMENTS IN THE MASS SPECTRA OF PARTIALLY METHYLATED D-GLUCITOL PERACETATES AND D-GLUCITOL HEXAACETATE

| *Compound** | *m/z* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *47* | *73* | *117* | *145* | *161* | *189* | *217* | *233* | *261* | *277* | *289* | *305* | *333* |
| 2,3,6-$OCH_3$ | ○ | ○ | ○ | | ○ | ○ | | ○ | | ○ | | ○ | |
| 2,6-$OCH_3$ | ○ | ○ | ○ | | | ○ | | | ○ | | | ○ | ○ |
| 3,6-$OCH_3$ | ○ | ○ | ○ | ○ | | ○ | | ○ | ○ | | | ○ | ○ |
| 2,3-$OCH_3$ | | ○ | ○ | ○ | ○ | | ○ | ○ | ○ | | | ○ | |
| 6-$OCH_3$ | ○ | ○ | ○ | ○ | | ○ | ○ | | ○ | | ○ | | ○ |
| 2-$OCH_3$ | | ○ | ○ | ○ | | ○ | ○ | | ○ | | ○ | | ○ |
| 3-$OCH_3$ | | ○ | | ○ | | ○ | ○ | | ○ | | | | ○ |
| Hexaacetate | | ○ | | ○ | | | ○ | | | | ○ | | |

* Abbreviations as in Table I.

The combinations of primary fragments in the mass spectra of these partially methylated D-glucitol acetates and D-glucitol hexaacetate[6] were useful for the unambiguous assignments of peaks in the gas chromatogram (Table II).

*Fragmentation analysis*

The methylation product prepared from β-CD by method B was successively hydrolysed, reduced and acetylated. The mixture of resulting partially methylated D-glucitol peracetates was subjected to GC (Fig. 5). Determination of the percentage

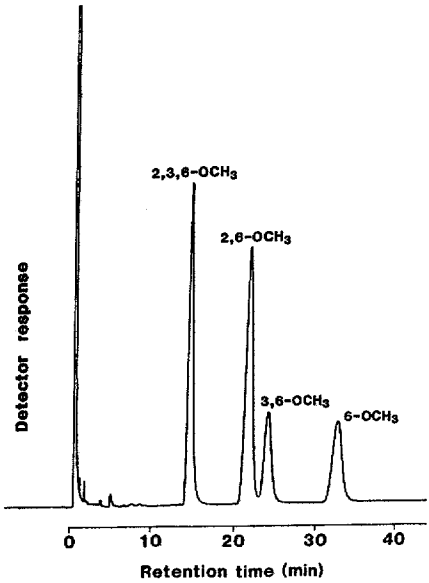



Fig. 5. Gas–liquid chromatogram of partially methylated D-glucitol peracetates derived from the methylation product of β-CD prepared by method B. Abbreviations and chromatographic conditions as in Table I.

composition by a digital electronic data processor resulted in 30% of 2,3,6-$OCH_3$, 40% of 2,6-$OCH_3$, 12% of 3,6-$OCH_3$ and 16% of 6-$OCH_3$, and at a higher sensitivity trace amounts of 2,3-$OCH_3$, 2-$OCH_3$, 3-$OCH_3$ and hexaacetate were also detected. Consequently, it was concluded that method B is not a selective methylation method.

Only two peaks (5% of 2,3,6-$OCH_3$ and 95% of 2,6-$OCH_3$) were detected in the gas chromatogram of partially methylated D-glucitol peracetates derived from the methylation product of β-CD synthesized by method A. Attainment of quantitative recoveries of methylated sugars is generally difficult as some demethylation during the hydrolysis and some losses of the more volatile methylated sugars during concentration are unavoidable. Hence the actual percentage of 2,3,6-$OCH_3$ may be higher than the observed value. This result shows that methylation by method A of $C_6$-OH and $C_2$-OH proceeds in preference to that of $C_3$-OH, but before complete methylation of the former, partial methylation of the latter begins. Szejtli *et al.*[3] reported that during the addition of the reagent it was advisable to maintain the temperature below 20°C in order to prevent the methylation of $C_3$-OH. In our experiments, however, holding the temperature below 20°C could not prevent the production of 2,3,6-tri-O-methyl ether. In this instance, 9.6-fold amounts of reagent required for methylation of all the $C_6$-OH and $C_2$-OH were used following Szejtli *et al.*'s method[3]. Further, β-CD was methylated according to Boger *et al.*[2] with 3.5-fold amounts of reagent at 0°C under nitrogen, but the ratio of 2,6-$OCH_3$ and 2,3,6-$OCH_3$ in the product was almost unchanged and the amounts of undermethylation products, having smaller $R_F$ values on TLC and shorter $t_R$ values on $C_{18}$-bonded silica, increased. The time course of methylation by method A revealed that a reaction time of 3 h was sufficient (lit.[3], 4 days).

*Isolation and characterization of partially methylated β-CD*

Three partially methylated β-CD derivatives (I, II and III) corresponding to the peaks 1, 2 and 4 in Fig. 4, respectively, were isolated by semi-preparative HPLC on a Nova-Pak $C_{18}$ cartridge column eluted with 2-propanol–water (19:81).

The pure samples of I, II and III were successively hydrolysed, reduced and acetylated, and the resulting products were characterized by GC–MS (Table III). There was only one product from I, 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol (2,6-$OCH_3$). The products from II and III contained 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol (2,3,6-$OCH_3$) in addition to 2,6-$OCH_3$ with 2,3,6-$OCH_3$:2,6-$OCH_3$ ratios of 1:6 for II and 2:5 for III. These results indicate that I is heptakis(2,6,-di-O-methyl)-β-CD, II is hexakis(2,6-di-O-methyl)-mono(2,3,6-tri-O-methyl)-β-CD and

TABLE III

YIELDS (%) OF PARTIALLY METHYLATED D-GLUCITOL PERACETATES DERIVED FROM COMPOUNDS I, II AND III

| *Product** | *I* | *II* | *III* |
|---|---|---|---|
| 2,6-$OCH_3$ | 100 | 86 | 72 |
| 2,3,6-$OCH_3$ | 0 | 14 | 28 |

* Abbreviations as in Table I.

III is pentakis(2,6-di-O-methyl)-bis(2,3,6-tri-O-methyl)-β-CD (one of the three isomers).

Their molecular weights were confirmed by FAB-MS analysis to be 1330 (I), 1344 (II) and 1358 (III). These data support the above-mentioned structures.

The physical and analytical data for the pure heptakis(2,6-di-O-methyl)-β-CD are as follows: m.p. 276°C (decomp.); $[\alpha]_D^{23}$ +116.1° ($c$ = 1, $CHCl_3$); $[\alpha]_D^{23}$ +155.4° ($c$ = 1, $H_2O$); analysis, calculated for $C_{56}H_{98}O_{35} \cdot H_2O$, C 49.85, H, 7.47; found, C 49.67, H 7.51%.

ACKNOWLEDGEMENTS

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