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Isolation and characterization by NMR spectroscopy of three monosubstituted 4-sulfobutyl ether derivatives of cyclomaltoheptaose (β-cyclodextrin)

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Abstract

The substitution profile of 4-sulfobutyl ether derivatives of cyclomaltoheptaose (β-cyclodextrin) (SBE-β-CD) prepared in our laboratories has been previously described. However, in those studies, no attempt was made to characterize the positional or regional isomers of this material. SBE-β-CD derivatives with degrees of substitution of two or higher represent a large number of possible isomers dependent on this positional and regional substitution. The monosubstituted SBE derivative, however, cannot have regional isomers and, therefore, has only three possible substitution products related to the 2-, 3-, and 6-hydroxyl groups of a glucose unit. In this study the isomers were fractionated by preparative anion-exchange chromatography with the progress of the elution being followed by a capillary electrophoretic (CE) method that resolved these isomers. The eluent containing the isomers was processed, and the pure materials were characterized by nuclear magnetic resonance spectroscopy (^{1}H NMR, DEPT, HETCOR, HOHAHA). Through this analysis the assignment of the positional isomers was made. © 1997 Elsevier Science Ltd.

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1. Introduction

The objective of this study was to isolate and characterize the positional isomers of the monosubstituted 4-sulfobutyl ether derivative of cyclomaltoheptaose (β-cyclodextrin) (SBE-β-CD). The SBE-β-CD derivatives have been prepared for use as parenterally safe solubilizing and stabilizing agents [1–10]. These derivatives are a rather complex mixture of various degrees of substitution (ds) depending on the reaction

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conditions. The substitution profile of the mixtures has been previously described [11–13]; however, in those studies no attempt was made to characterize the positional or regional isomers of these materials. Furthermore, an understanding of the simpler monosubstituted derivative was thought to provide valuable insight into more complex mixtures such as $(SBE)_{4m}$-β-CD or $(SBE)_{7m}$-β-CD and to form the basis for positional and regional characterization.

The SBE-β-CD derivatives with ds of two or more are mixtures of a great number of substituted analogs containing regio and positional isomers in each level of substitution. Only three positional isomers related to the 2-, 3-, and 6-hydroxyl groups of a glucose unit of the β-CD are possible with the monosubstituted material. The structure of one of these isomers is presented in Fig. 1, although the substitution could be in either the 2-, 3-, or 6-position in a glucose unit.

A capillary electrophoresis (CE) method was developed that resolved the $(SBE)_{1b}$-β-CD (where **b** 'band' refers to the mixture of isomers) into three peaks as can be observed in Fig. 2. The three peaks observed under these conditions were arbitrarily named **A**, **B**, and **C** following their CE elution order, having **A** the peak with the shortest migration time.

Evidence of the presence of the three possible isomers of the $(SBE)_{1b}$-β-CD was also initially shown by nuclear magnetic resonance (NMR) spectroscopy. However, one- and two-dimensional NMR studies of the band were complex due to a breakdown of the molecular symmetry precluding the investigation of positional isomers and specific assignment of each of these positional isomers without isolation of at least one or two of them. Thus, fractionation of the monoband was necessary.

The present work describes the fractionation of the $(SBE)_{1b}$-β-CD by preparative anion-exchange chromatography (AEC), with the elution being followed by CE analysis, and subsequent NMR spectral characterization of the isolated isomers.

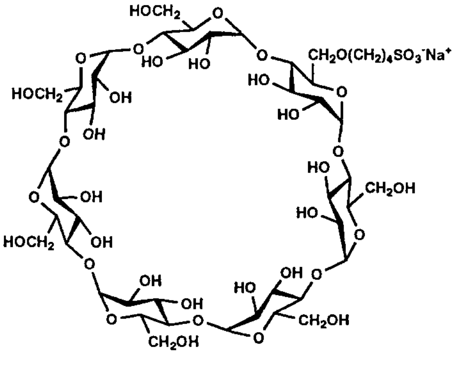

Fig. 1. Structure of 6-*O*-sulfobutyl ether of cyclomaltoheptaose (β-cyclodextrin) (the 6-*O*-SBE-β-CD).

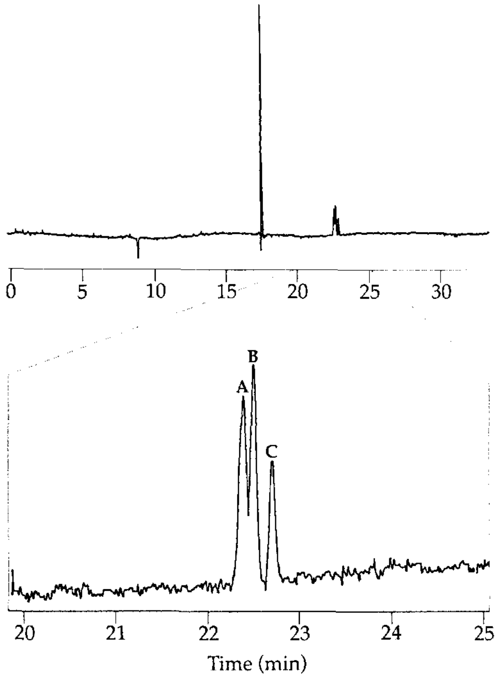

Fig. 2. Electropherogram of a 0.8 mM solution of $(SBE)_{1b}$-β-CD with an expanded view of the 20–25 min region. Conditions: modular CE; uncoated capillary, 50 μm i.d., 365 μm o.d., 120 cm total length, 90 cm effective length; field strength, 167 $V \cdot cm^{-1}$; running buffer, 15 mM benzoic acid–Tris, pH 6.0; detection: indirect UV 230 nm; sample introduction, 1 s at 5 psi; temperature of separation, ambient.

2. Results and discussion

Anion - exchange chromatography (AEC).—The fractionation of a mixture of SBE-β-CD in the different levels of substitution by AEC using DEAE-Sephadex A-25 has been previously reported [12]. These results suggested that the elution could be controlled by the use of certain selected concentrations of the mobile phase. Therefore, this methodology was extended to attempt the fractionation of $(SBE)_{1b}$-β-CD.

(a) Weak anion-exchange fractionation. — In a preliminary experiment, a low resolution of the isomers was obtained with sodium sulfate at pH 5.5. This separation was further improved when the pH of the mobile phase was decreased from 5.5 to 3.5, which may have increased the number of charged groups in the matrix and, therefore, its exchange capacity. Fig. 3 depicts the elution profile obtained for the three positional isomers eluted with Na_2SO_4 at pH 3.5.

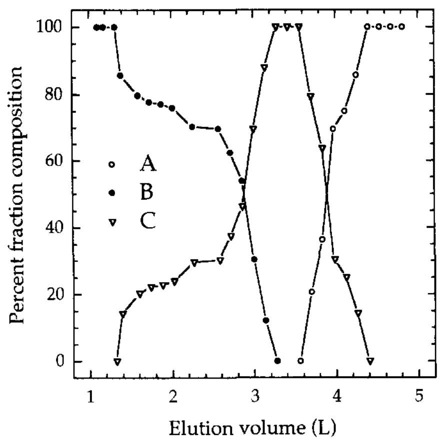

Fig. 3. Elution profile obtained for the fractionation of $(SBE)_{1b}$-β-CD using weak anion-exchange resin. Experimental conditions: bed height, 3.5 cm i.d. × 31 cm; mobile phase, step gradient elution 1–2 mM Na_2SO_4; flow rate, 0.5 mL/min; sample loaded 1.4 g (mixture composition depicted in Fig. 2); fraction size, 9 mL.

DEAE-Sephadex resin is a cross-linked dextran matrix that is highly hydrophilic. The ion-exchange group is diethylaminoethyl (a weak anion-exchanger), and the major separation mechanism resolves species according to differences in charge [14]. The group bearing the charge in the SBE moiety is separated from the covalent link to the β-CD torus, and no differences in the ionization state of the three isomers should be expected, particularly at the pH of the experiments. Therefore, it seems unlikely that the ion-exchange process alone is responsible for the separation observed (Fig. 3). Dextran is a cellulose polymer and structural similarities between the Sephadex matrix and SBE-β-CD suggest that some adsorption also may occur.

A major practical disadvantage of this procedure was the very slow flow rates that were encountered. To overcome this problem it was necessary to identify a commercial resin that allowed faster flow rates.

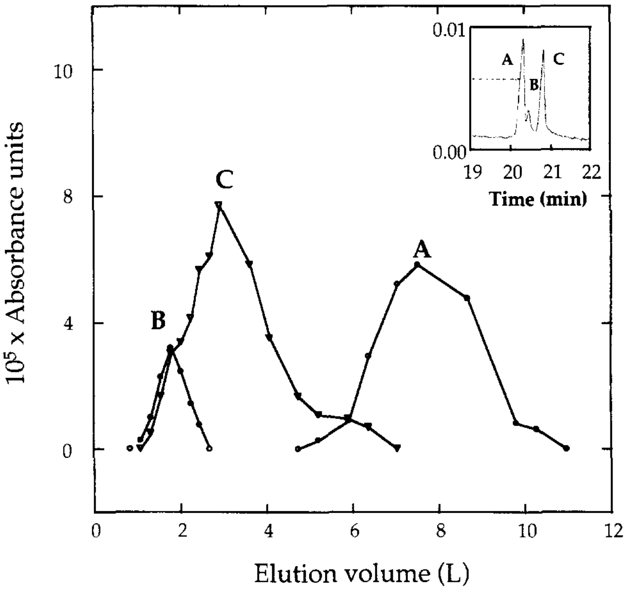

Fig. 4. Elution profile obtained for the fractionation of $(SBE)_{1b}$-β-CD using strong anion-exchange resin (Dowex-1) under the following conditions: bed height, 2.4 cm i.d. × 30 cm; mobile phase, 5 mM NaCl; flow rate, 4 mL/min; sample loaded, 2.8 g with a mixture composition as depicted in electropherogram included in the inset figure (CE conditions: P/ACE 2210; uncoated capillary, 50 μm i.d., 365 μm o.d., 107 cm total length, 100 cm effective length; field strength, 280 $V \cdot cm^{-1}$; running buffer, 30 mM benzoic acid–Tris, pH 6.0; detection: indirect UV 230 nm; sample introduction, 1 s at 5 psi; temperature of separation, 25 °C.

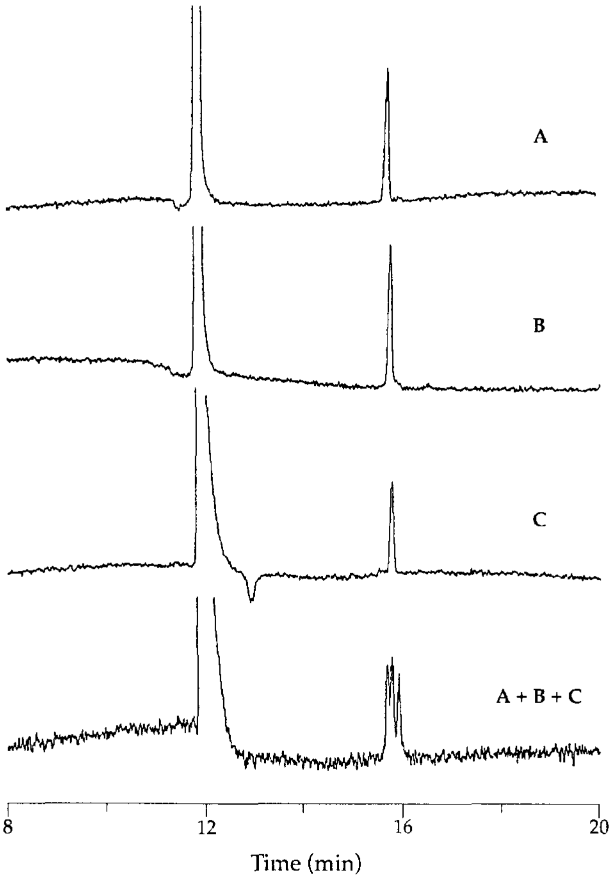

Fig. 5. Electropherograms of the isolated compounds **A** (2-*O*-SBE-β-CD), **B** (6-*O*-SBE-β-CD), and **C** (3-*O*-SBE-β-CD) and a mixture **A**, **B**, and **C** (bottom). Conditions: P/ACE 2210; uncoated capillary, 50 μm i.d., 365 μm o.d., 50 cm total length, 57 cm effective length; field strength, 167 $V \cdot cm^{-1}$; running buffer, 30 mM benzoic acid–Tris, pH 6.0; detection: indirect UV 230 nm; sample introduction, 1 s at 5 psi; temperature of separation, 25 °C; sample concentration, 1 mg/mL.

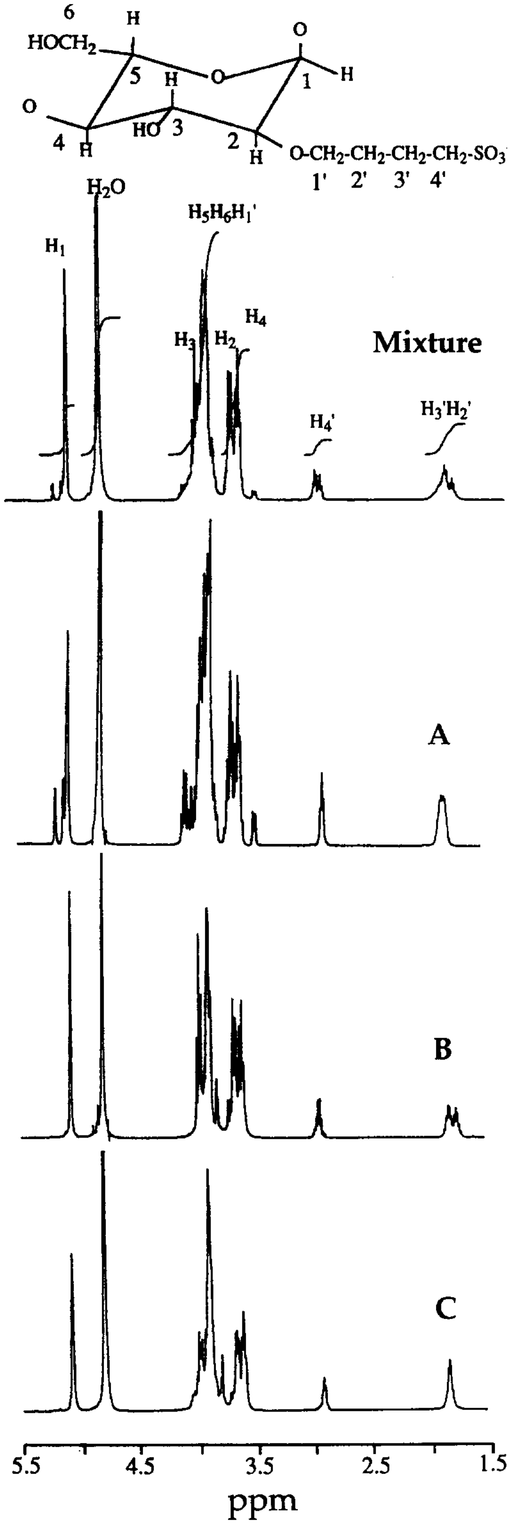

Fig. 6. ^{1}H NMR spectra of the $(SBE)_{1b}$-β-CD mixture (top) and the isolated compounds: **A** (2-*O*-SBE-β-CD), **B** (6-*O*-SBE-β-CD), and **C** (3-*O*-SBE-β-CD).

(b) Strong anion-exchange fractionation. — Fig. 4 depicts the elution profile obtained for the fractionation of the $(SBE)_{1b}$-β-CD on a strong anion-exchange resin (Dowex-1). A CE profile of the $(SBE)_{1b}$-β-CD loaded on the column is provided in the Figure (inset).

Dowex-1 is a polymeric based resin (styrene–divinylbenzene) with a quaternary amine exchange moiety. The working pH range for this resin is 0–14, which means the resin is at its maximum ion exchange capacity without having to adjust the pH of the mobile phase.

In a preliminary experiment, a low resolution of the isomers was obtained with 2 mM sodium sulfate. This counterion easily displaced the compounds from the ion-exchange site. A better resolution was obtained with sodium chloride in the mobile phase when compared to sodium sulfate. Although, the separations of the isomers with sodium chloride showed no improvements when compared to the fractionation on Sephadex at pH 3.5 with sodium sulfate, the overall fractionation was accomplished six times faster.

Capillary electrophoresis analysis.—Under the general conditions for the analysis of the SBE-β-CDs mixtures with average ds greater than one [11,12],

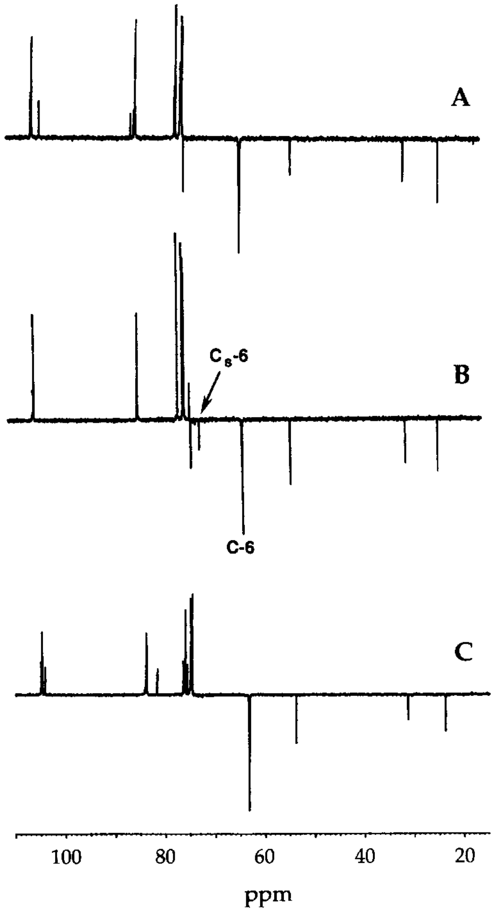

Fig. 7. DEPT spectra of the isolated compounds: **A** (2-*O*-SBE-β-CD), **B** (6-*O*-SBE-β-CD), and **C** (3-*O*-SBE-β-CD).

$(SBE)_{1b}$-β-CD migrates as a single peak. Modification of the conditions by a combination of a decrease in the field strength and an increase in the length of the capillary resolved the band into the three isomers as shown in Fig. 2.

Fig. 5 demonstrates the purity of the solids isolated using the weak anion-exchange resin. Although the isomers could not be identified based on the migration times, a synthetic mixture of equal volumes of the three solutions of compounds **A**, **B**, and **C** was clearly resolved into three peaks (Fig. 5, bottom electropherogram). The identity of the peaks was established by spiking each of the samples with a mixture of the three isomers with known composition.

Structure elucidation.—The ^{1}H NMR spectra of the monosubstituted cyclodextrins are relatively uninformative (Fig. 6). The H-1 protons are well separated, but all of the other 42 carbohydrate protons overlap extensively, even in two dimensions at 500 MHz, in the region between δ 3.5 and 4.1. The protons resonated in the order H-3 < H-5 ~ H-6 < H-2 ~ H-4 (from downfield to upfield). The ^{13}C NMR spectrum (DEPT) was easier to analyze (Fig. 7) because the seven glucose units are equivalent. C-1, the anomeric site, is well separated downfield shift (δ 104) while C-4, the site of the glycosidic linkage, is also shifted downfield, and the C-6, the only methylene carbon, is well upfield at δ 64. However, C-2, -3, and -5 occur within a narrow area around δ 75.

In all three cases, addition of the SBE group adds four new methylene carbon resonances and breaks each of the formerly equivalent carbohydrate peaks into an overlapping band of nonequivalent peaks, while shifting the carbon with the ether linkage downfield about 8 ppm. In the 6-isomer only this carbon is a methylene (C_s-6) and is readily identified at δ 72 in the DEPT spectrum of compound **B** (Fig. 7, middle spectrum). The methylene signals of the SBE side chain are also negative. In spectrum C the C-4′ signal near δ 75 is obscured by overlap with more intense methyne signals.

However, for the 2- and 3-isomers, the affected carbon will be one of many methynes, and the shift in

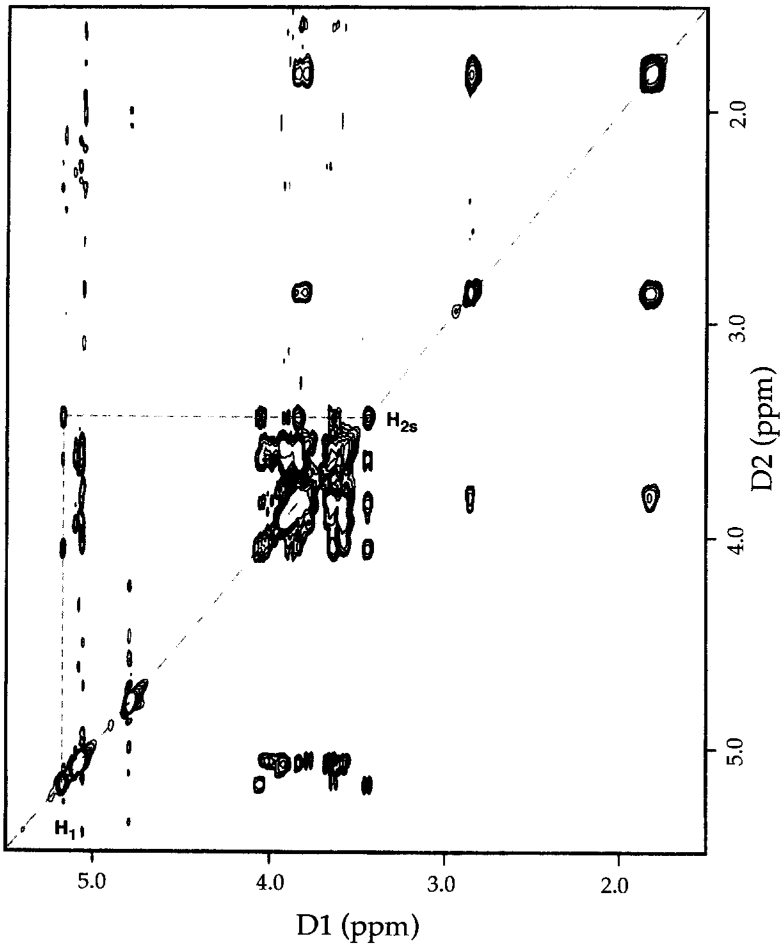

Fig. 8. HOHAHA spectrum of compound **A** (2-*O*-SBE-β-CD).

derivatization will put it in the vicinity of the C-4 envelope. Unambiguous identification of whether it is the C-2 or C-3 peak that has lost one-seventh of its intensity is difficult. Hence additional information must be sought from two-dimensional spectra to assign these two isomers.

In the ^{1}H NMR spectrum of compound **A** (Fig. 6) only, the H-1 signal is split into three peaks with integrals 1:1:5. The most downfield (integral 1) is on the substituted glucose; the next (only slightly shifted downfield) is on an adjacent glucose; the other five are essentially indistinguishable. The most downfield of the H-1 peaks can be seen in the HOHAHA spectrum (Fig. 8) to couple to an H-2 peak which is very upfield at δ 3.45. In the HETCOR spectrum (Fig. 9, compound **A**, top) this proton is connected to a C-2 resonance shifted downfield to δ 84, which demonstrates this fraction is the 2-*O*-SBE isomer. As in the proton spectrum, the C-1 signals are perturbed more in this isomer than in the other two; the C-1 adjacent to the point of substitution is shifted 2 ppm upfield from the other six signals.

The ^{1}H NMR spectrum of compound **C** (Fig. 6, bottom), which must be the 3-*O*-SBE isomer, gives no such characteristic patterns, as the affected signals are within the overlapped region. However, in the HETCOR (Fig. 9, compound **C**, bottom) one of the carbons within the C-4 envelope is observed to correlate to an unusually downfield proton, suggesting that this is the derivatized C-3 (C_s-3). A single carbon resonance about 2 ppm upfield of this envelope is observed to correlate to a relatively upfield proton, suggesting this is the C-4 adjacent to the reaction site. Overall, it is concluded that at the reaction site the carbon is shifted downfield and the proton is shifted upfield. At the adjacent site the carbon signal is shifted upfield and the proton signal is shifted downfield. Based on these experiments the assignment of the CE peaks was as follows: **A** is the 2-*O*-SBE; **B** is the 6-*O*-SBE; **C** is the 3-*O*-SBE derivatized β-CD.

Chromatographic behavior of the isomers.—Although specific interactions with both resins, weak and strong, may be different, the elution order of the isomers was the same on both resins. The results suggest a common mechanism might be responsible for the fractionation of the isomers. The elution order observed (**B** = 6-*O*-SBE, **C** = 3-*O*-SBE, and then **A** = 2-*O*-SBE, see Figs. 3 and 4) suggests a stronger interaction for compound 2-*O*-SBE, and the interaction decreases in the order 2-*O*-SBE $\gg$ 3-*O*-SBE > 6-*O*-SBE. Although ion exchange might be the main separation mechanism, other interactions between the resin and the other portions of the cyclodextrin derivatives may have contributed to the resolution of the compounds.

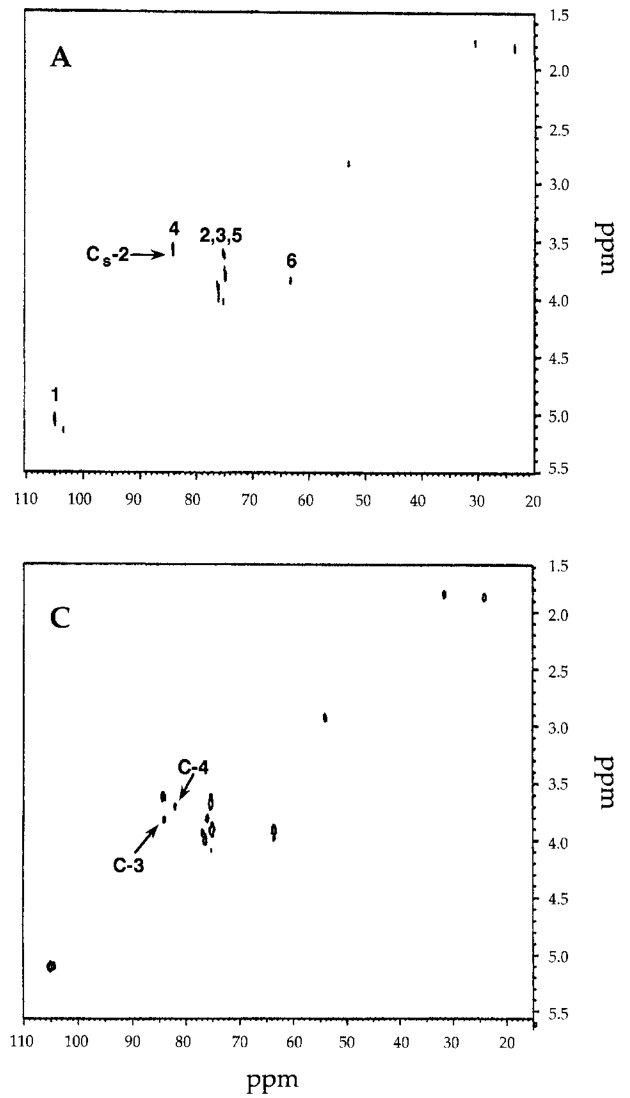

Fig. 9. HETCOR spectra of compounds **A** (2-*O*-SBE-β-CD) and **C** (3-*O*-SBE-β-CD).

Electrophoretic behavior of the isomers.—Under the CE conditions, with benzoate ions in the running buffer, the negatively charged species migrate electrophoretically opposite to the detector, while the intense electroosmotic flow reverses the direction of the apparent mobility. Obviously, the interaction of the isomers with the benzoate ion is different, giving a change in their relative mobilities, thereby allowing separation of the isomers. A faster migrating isomer indicates that the benzoate interacts less strongly with that isomer than with the others. Making this assumption, the interaction for these isomers increases in the order 2-*O*-SBE < 6-*O*-SBE $\ll$ 3-*O*-SBE.

3. Conclusion

In summary, the positional isomers of $(SBE)_{1b}$-β-CD were successfully fractionated by preparative anion-exchange chromatography with the progress of the elution being followed by a capillary electrophoretic (CE) method. The pure materials were characterized and the substitution site assigned by 2D NMR experiments. The NMR spectra of the isomers provided valuable insight that will hopefully allow positional isomers assignments for more complex mixtures such as $(SBE)_{4m}$-β-CD and $(SBE)_{7m}$-β-CD.

4. Experimental

Materials.—All chemicals used were of at least analytical reagent grade purity. Na_2SO_4 and NaCl were purchased from Aldrich Chemical Company (Milwaukee, WI). Benzoic acid, tris(hydroxymethyl)aminomethane (Tris) and Dowex-1 were obtained from Sigma Chemical (St. Louis, MO). The preparation and isolation of $(SBE)_{1b}$-β-CD was previously reported [1,2,12]. The capillary column was purchased from PolyMicro Technologies (Phoenix, AZ). DEAE-Sephadex A-25 was obtained from Pharmacia LKB (S-75182, Uppsala). Diaflo membranes were obtained from Amicon (YC5, 75 mm, AMICON, Beverly, MA).

NMR spectroscopy.—All spectra were taken on a Bruker AM 500 instrument operating at 500.14 MHz for ^1H, equipped with broadband and inverse detection probes. DEPT, HOHAHA, and HETCOR experiments were performed with standard Bruker pulse programs.

Capillary electrophoresis (CE) analysis.—The capillary electrophoresis analyses were carried out on a Beckman P/ACE 2210 (Beckman Instruments, Fullerton, CA) CE or on a modular laboratory built electropherograph. New columns were conditioned by filling the capillary with 0.1 N NaCl, soaking for 20 min, and rinsing with 5 vol exchanges (ve) of water. Sodium hydroxide (0.1 N) was prepared and stored in a glass bottle. Double distilled water was used to prepare the solutions.

Conditions for the P/ACE 2210 CE.—The washing sequence previous to the injection was at 20 psi: 3 min 0.1 N sodium hydroxide, 1 min water and 2 min running buffer. The buffer at both ends of the capillary was replaced every three runs. The running buffer was 30 mM benzoic acid–Tris pH 6.0 that was prepared following the procedure previously reported [12].

Conditions for the modular CE.—The modular CE was previously described [11,12]. The system consisted of a Spellman Model CZE 1000R high-voltage power supply (Plainview, NY) and an ISCO Model CV4 UV-vis detector (Lincoln, NE). The running buffer (15 mM benzoic acid–Tris) was prepared from the 30 mM benzoic acid–Tris buffer. The capillary was treated with the following wash sequence before each injection: 7 vol exchanges (ve) of distilled water, 5 ve 0.1 N sodium hydroxide, 3 ve water, 5 ve running buffer, baseline equilibration 5 min. The buffer at both ends of the capillary were replaced every three runs. Specific experimental conditions are included in the figure captions.

Anion - exchange chromatography (AEC).—The $(SBE)_{1b}$-β-CD was fractionated by anion-exchange chromatography following the general procedure previously described [12]. The fractionation was accomplished independently by using a weak and a strong anion-exchange resin.

(a) Weak anion-exchange fractionation. — The activation procedure of the DEAE-Sephadex A-25 and the column preparation were previously described [13]. The composition of this sample is depicted by the CE profile of Fig. 2 and has relative ratios of **A**, **B**, **C**, calculated from the peak area in the electropherogram, of approximately 1.8:1.7:1, respectively. The initial mobile phase was 1 mM Na_2SO_4 at pH 3.5 and then increased to 2 mM pH 3.5. The $(SBE)_{1b}$-β-CD mixture was loaded as a concd aq soln with a small pipette, and the elution was by gravity flow at a constant solvent height of 30 cm.

The fractions were homogenized, and an aliquot was analyzed by CE without further treatment. The peaks were identified by addition of a standard mixture of $(SBE)_{1b}$-β-CD and observing the change in peak height or peak area ratio. The percent fraction composition was used to construct the elution profile of each experiment, and the results are shown in Fig. 3. The fractions containing pure compound were pooled, neutralized to pH 7.0 with 0.1 N sodium hydroxide, and concd under reduced pressure at 50 °C to approximately 100 mL. The resulting soln was then transferred to a 400-mL-ultrafiltration cell (AMICON, ultrafiltration stirred cell, model 8400), diluted to 150 mL and dialyzed through a Diaflo cellulose membrane previously activated with double-distilled water during 1 h period, replacing the water in approximately 15 min intervals. The desalting was performed at 10–15 psi and monitored until an aliquot of the filtrate tested negative for sulfate to the addition of 0.5% w/v barium chloride pH 2.0. The filtrate was discarded, and the resulting retentate was filtered through a 0.22 μm nylon filter, concd to approximately 10–15 mL and lyophilized. The yield based on the initial mass loaded onto the column was 28% of **A**, 3% of **B**, and 5% of **C**. Fractions containing mixtures of **B** and **C** were processed to account for the mass balance to yield 597 mg. The total percent mass recovered was 79%.

(b) Strong anion-exchange fractionation. — The resin used for the fractionation was Dowex-1, and the experiment was conducted as described below. The mobile phase was prepared by dissolving the corresponding amount of sodium chloride in 1 L of water.

The resin was soaked for 24 h at room temperature with 5% w/v of NaCl with occasional stirring, and the supernatant was changed 3 or 4 times with fresh soln. The resin was packed into the column as a slurry, and the resin bed was conditioned for 2 h at a fast flow rate and 20 h at a very slow flow. The column was then washed with double-distilled water until it tested free of chloride by 0.1 N silver nitrate soln.

The sample was loaded as a concd aq soln (2.8 g of $(SBE)_{1b}$-β-CD in 40–50 mL). The pattern of composition of the mixture obtained by capillary electrophoresis is included in Fig. 4 (inset, conditions described in the legend), and the relative ratios, calculated from the peak area in the electropherogram, were approximately 4.9:1:3.6 of **A**, **B**, and **C**, respectively. The mobile phase was 5 mM NaCl with a flow rate of 3 mL/min. The fractionation was conducted at room temperature. The fractions collected were analyzed by CE in the P/ACE 2210 system as described above. The fractions containing the same peak (corroborated by spiking with the $(SBE)_{1b}$-β-CD) were pooled and concd at reduced pressure at 50 °C to approximately 50 mL, ultrafiltered, filtered, and lyophilized as described above. The progress of the ultrafiltration was monitored with 0.1 N silver nitrate. No attempt was made to recover the material that went through the cellulose membrane. The yield based on the original amount loaded onto the column was 36% and 21% of pure **A** and **C**, respectively. Fractions containing mixture of **B** and **C** or **C** and **A** were not processed. The results are summarized in Fig. 4.

Acknowledgements

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