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Fractionation and characterization of 4-sulfobutyl ether derivatives of cyclomaltoheptaose (β-cyclodextrin)

Ernestina A. Luna [a], Esteban R.N. Bornancini [a], Diane O. Thompson [b], Roger A. Rajewski [a,*], Valentino J. Stella [a]

[a] *Center for Drug Delivery Research, Higuchi Biosciences Center, University of Kansas, 2099 Constant Ave., Lawrence, KS 66047, USA*

[b] *CyDex, L.C., 7304 West 130th Street, Suite 370, Overland Park, KS 66213, USA*

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Abstract

4-Sulfobutyl ether derivatives of cyclomaltoheptaose (β-cyclodextrin) (SBE-β-CD) are being developed as parenterally safe solubilizing and stabilizing agents. SBE-β-CDs are a mixture of positional and regional isomers containing from one to as many as twelve sulfobutyl ether (SBE) groups per cyclodextrin. Capillary electrophoretic (CE) analysis of these mixtures resolves these isomers based on the molar degree of SBE substitution (ds), and the electropherogram shows an almost symmetrical distribution of SBE incorporation centered around the band which represents the apparent average degree of substitution for the mixture. The objectives of this study were to isolate the different substitution bands for their characterization and to evaluate their mass contribution to the mixture. Mixtures of SBE-β-CDs containing from mono- up to deca-SBE substitutions were fractionated by preparative anion-exchange chromatography with salt concentration gradient elution. The bands for each ds were well resolved as characterized by CE analysis with indirect UV detection. The isolated materials were desalted and lyophilized to obtain white solids, which were then characterized by nuclear magnetic resonance (^{1}H NMR) spectroscopy, capillary electrophoresis (CE), and fast-atom-bombardment mass spectrometry (FABMS). The CE molar response factor of each ds was then determined, and the actual percent mass composition of a SBE-β-CD mixture was calculated. © 1997 Elsevier Science Ltd.

Keywords: Anion-exchange chromatography; 4-Sulfobutyl ether cyclomaltoheptaose (cyclodextrin); Anionic cyclodextrin

* Corresponding author.

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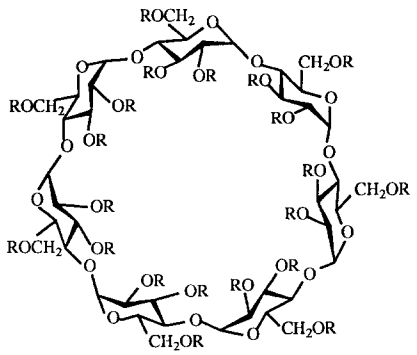
1. Introduction

The overall objective of this study was to fractionate a mixture of 4-sulfobutyl ether derivatives of cyclomaltoheptaose (β-cyclodextrin) (SBE-β-CD) for better characterization of the materials. The recently patented SBE-β-CD is currently being developed as a parenterally safe solubilizing agent [1–3]. These derivatives have been shown to have potential applications as a solubility and stability enhancer, as well as chiral-selective analytical reagents [1,2,4–10].

The SBE-β-CD mixtures are the product of the reaction of 1,4-butane sultone with β-CD in an alkaline solution [1,2]. Since the introduction of the SBE moiety may occur on any of the 21 available hydroxyl sites of β-CD, a large number of positional isomers within the cyclodextrin framework are possible, and, furthermore, homologous derivatives with lower or higher degrees of substitution are also formed in addition to the product [11]. Thus, the product mixture includes regio and positional isomers distributed over a range of substitution levels. These mixtures are generally characterized by an average degree of substitution (ds), calculated on the basis of elemental analysis (ds_{EA}) and/or nuclear magnetic resonance (ds_{NMR}) data. The ds expresses the average number of substituted hydroxyl groups per cyclodextrin molecule. A generalized structure of the SBE-β-CD derivatives is provided in Fig. 1.

The SBE-β-CD mixtures can be reproducibly prepared with different average ds (i.e., 1, 4, 7) [12,13] by varying the reaction conditions. Capillary electrophoresis (CE) has proved to be a useful analytical tool to show differences in the composition of the



R = -$CH_2CH_2CH_2CH_2SO_3Na$ or -H

Fig. 1. Generalized structure of the sulfobutyl ether β-cyclodextrin sodium salt, $(SBE)_{nm}$-β-CD, where n is the average degree of substitution (ds) and m stands for mixture of different ds.

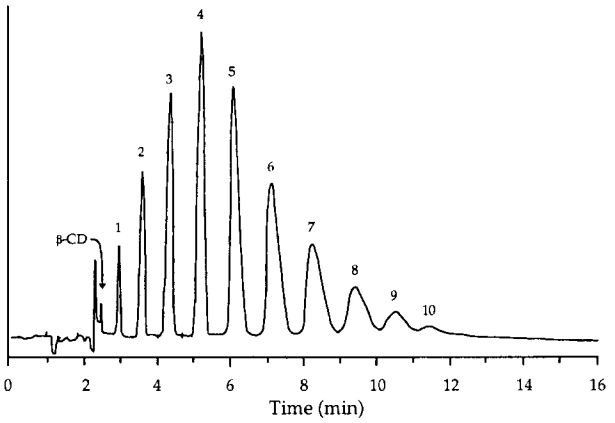


Fig. 2. Electropherogram obtained with a mixture of $(SBE)_{4m}$-β-CD under the following conditions: uncoated capillary, 50 μm i.d., 365 μm o.d., 57 cm total length, 50 cm effective length; field strength, 417 V cm^{-1}; running buffer, 30 mM benzoic acid–Tris, pH 6.0; indirect detection, 230 nm; sample introduction, 5 s at 0.5 psi; temperature of separation, ambient.

various SBE-β-CD mixtures [12,13]. Fig. 2 illustrates a typical electropherogram of a $(SBE)_{4m}$-β-CD preparation. The CE profile depicts the mixture resolved in eleven peaks with the fastest eluting compound corresponding to unreacted β-CD. The next two peaks (1 and 2 in Fig. 2) have been previously identified as corresponding to SBE derivatives with a defined ds of 1 and 2, respectively [12]. This information together with fast-atom-bombardment mass spectrometry (FABMS) of the mixtures allowed the theoretical assignment of the peaks with increasing ds. Each of these peaks (bands) should correspond to a mixture of isomers bearing the same number of charges per CD molecule. However, the identity of the peaks with ds > 2 needs to be confirmed. An average ds based on the percent contribution of each peak to the total peak area composition of a CE profile (ds_{CE}) can also be calculated, but the value obtained in this way does not agree with the ds_{EA} and ds_{NMR}.

Although the FABMS spectra and CE analysis produce a distribution profile of the SBE-β-CD mixtures, and EA and NMR data provide an average ds, none of these methods gives information regarding the individual mass contribution of the different ds of the mixture. Therefore, in this work we present the fractionation of a SBE-β-CD mixture by anion-exchange chromatography (AEC) into the different ds; the characterization of the bands by NMR, CE, and FABMS; and finally, the determination of the CE molar response factor of each substitution band that allowed estimation the actual mass contribution to a given SBE-β-CD mixture.

2. Results and discussion

Fractionation of the SBE-β-CD mixture.—Given the anionic characteristics of the cyclodextrin derivatives, AEC was a reasonable approach for the fractionation of the SBE-β-CD.

Fig. 3 depicts the AEC elution profile obtained in the fractionation of SBE-β-CD mixtures and the sodium sulfate gradient used to elute the compounds. The mixture was well resolved at each substitution level as analyzed by CE. Sodium sulfate, which is one of the strongest salts for weak anion-exchange resins [14], has a relatively low molecular weight compared to the CDs, allowing removal by ultrafiltration, and its presence can be easily monitored by visualization with barium chloride. The first part of the fractionation was performed at relatively low pH (3.5–4.0), and after elution of the band corresponding to CE peak number 4 the pH of the mobile phase was no longer adjusted, thereby facilitating the elution of the highly charged CDs by decreasing the number of active sites (due to partial neutralization of the protonated DEAE groups). As expected, β-CD was not retained under the experimental conditions and eluted with the solvent front in the first 500 mL collected. Since the group bearing the charge in the SBE moiety

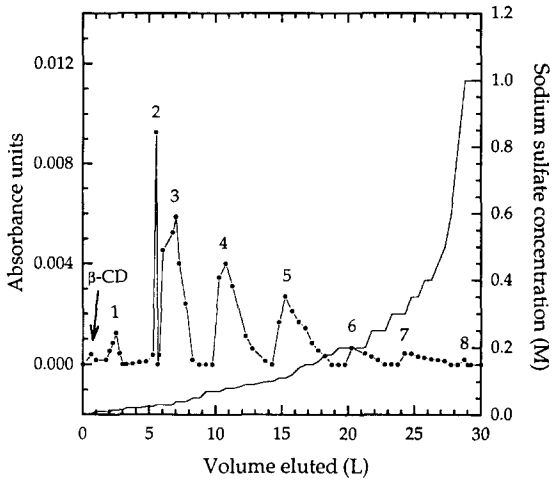


Fig. 3. Elution profile obtained for the fractionation of the $(SBE)_{4m}$-β-CD by anion-exchange chromatography under the following conditions: packing material, DEAE Sephadex A-25; dimensions, 28 cm×4.1 cm i.d. bed height; fraction size, 125 mL; mobile phase, sodium sulfate gradient concentration. Capillary electrophoresis conditions for the analysis of the fractions: uncoated capillary, 50 μm i.d., 365 μm o.d., 57 cm total length, 50 cm effective length; field strength, 526 V cm^{-1}; running buffer, 23 mM benzoic acid–25 mM Tris, pH 7.0; indirect detection, UV 230 nm; sample introduction, 5 s at 0.5 psi; temperature of separation, 25 °C.

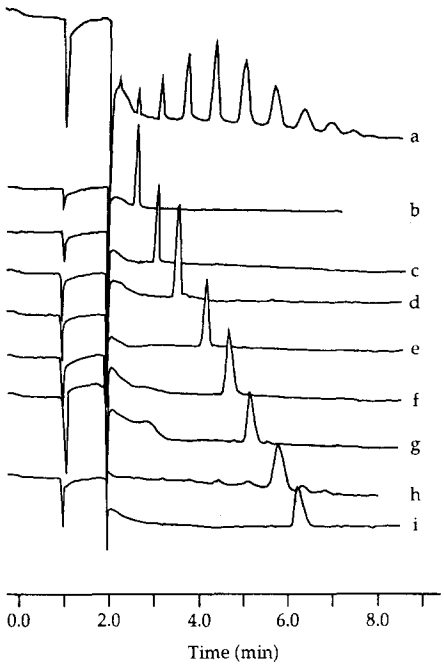


Fig. 4. Capillary electrophoresis analyses of the SBE-β-CD isolated bands: a. $(SBE)_{4m}$-β-CD; b. $(SBE)_{1b}$-β-CD; c. $(SBE)_{2b}$-β-CD; d. $(SBE)_{3b}$-β-CD; e. $(SBE)_{4b}$-β-CD; f. $(SBE)_{5b}$-β-CD; g. $(SBE)_{6b}$-β-CD; h. $(SBE)_{7b}$-β-CD; i. $(SBE)_{8b}$-β-CD. The runs were performed under the capillary electrophoresis conditions of Fig. 3.

is removed from the cyclodextrin by a butyl chain, it seems very likely that the ion-exchange process alone is responsible for the separation obtained.

The ultrafiltration step proved to be time consuming, but slow flow rates and dilute solutions are recommended to decrease the loss of material through the membrane. This became a practical problem for the highly substituted material where the salt content was high, as it took several weeks for the complete desalting of the samples. To overcome this difficulty, the difference in water solubilities of the SBE-β-CD and sodium sulfate was used to reduce the amount of salt. Although CE analysis of the precipitate from refrigerated solutions of the highly substituted bands (ds 5–8) showed the presence of SBE-β-CD (probably by occlusion into the crystals), no attempt was made to recover the material. The bands with ds 9 and 10 were not detected by CE or did not elute from the Sephadex under the experimental conditions.

Characterization of the SBE-β-CD bands.—After lyophilization, the isolated bands were analyzed by CE, ^1H NMR spectroscopy, and FABMS.

(a) CE characterization. Fig. 4 (b–i) depicts the CE profile obtained with each sample and the purity of the compounds as compared to the initial mixture [Fig. 4 (a)]. The CE peaks were assigned by compari-

son of their migration times, and the assignment was corroborated by spiking $(SBE)_{4m}$-β-CD with the respective bands. No additional peaks were observed in any of the electropherograms of the spiked samples. Although the electropherograms of the bands isolated show some impurities [i.e. Fig. 4 (d and g)], no attempt was made to further purify these compounds. The results demonstrate that eight of a total of ten initial components of the $(SBE)_{4m}$-β-CD were isolated.

(b) NMR characterization. The ^1H NMR spectrum of each band contained the same set of signals as the original $(SBE)_{4m}$-β-CD mixture (data not shown). The ^1H NMR of the SBE-β-CDs are rather complex

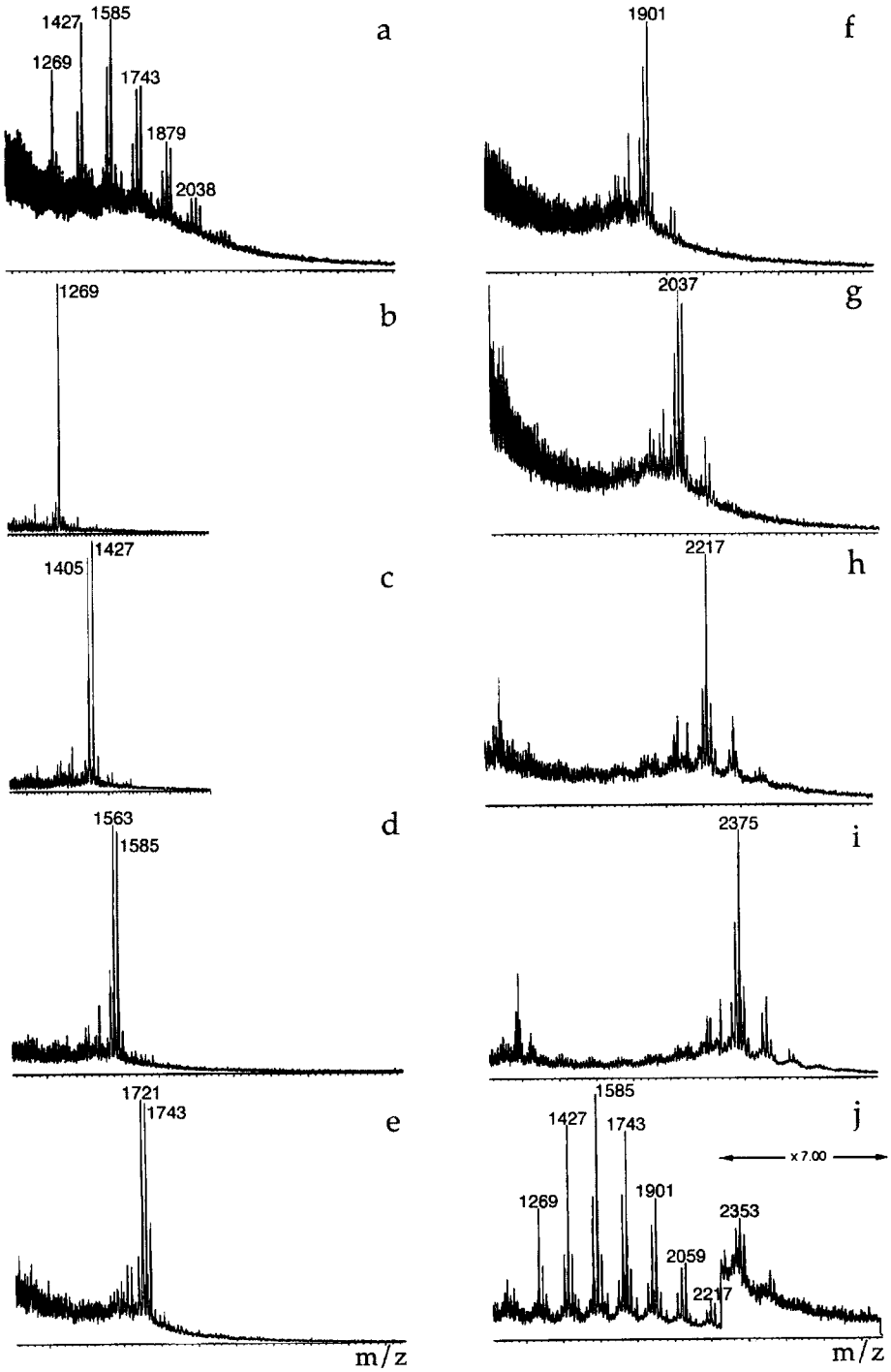


Fig. 5. FABMS of the SBE-β-CDs mixture and isolated bands recorded in negative-ion mode in glycerol (spectra a–g) and in triethanolamine (spectra h–j) matrix: a. $(SBE)_{4m}$-β-CD; b. $(SBE)_{1b}$-β-CD; c. $(SBE)_{2b}$-β-CD; d. $(SBE)_{3b}$-β-CD; e. $(SBE)_{4b}$-β-CD; f. $(SBE)_{5b}$-β-CD; g. $(SBE)_{6b}$-β-CD; i. $(SBE)_{7b}$-β-CD; h. $(SBE)_{8b}$-β-CD; i. $(SBE)_{4m}$-β-CD.

where the signals corresponding to the anomeric protons are well separated, but the other carbohydrate protons overlap extensively. Comparison of the spectrum of each band showed an evident growth of the signals corresponding to the methylenic protons of the SBE chain, relative to the anomeric protons of the cyclodextrin ring, which should be proportional to the amount of SBE groups in the sample. However, the ds calculated from the proton integration was erratic because the conditions for water suppression affected the area of the signals needed for the calculation. Acquisition of the ^{1}H NMR spectra without water irradiation may improve these results. Nevertheless the current data was sufficient to demonstrate that the skeleton of each of the SBE-β-CD bands isolated was intact after the fractionation process, since no additional signals possibly due to a breakdown of the molecule were observed, and the amount of methylene groups increased with the increase of the ds of each band.

(c) FABMS characterization. In order to explore the experimental conditions required to obtain the FABMS of each band, the spectrum of the $(SBE)_{4m}$-β-CD was obtained in two different matrices, glycerol (GLY; Fig. 5a) and triethanolamine (TEA; Fig. 5j). Fast-atom-bombardment in the negative-ion mode using TEA or GLY as a liquid matrix gave well-defined mass spectra and the molecular ions of the SBE-β-CDs were clearly separated. The FABMS of each band is presented in Fig. 5, where bands with ds 1–6 (Fig. 5b–g) were obtained in GLY and bands with ds 7 and 8 in TEA matrix (Fig. 5h and i). In each case the major peak corresponds to monoanions formed by the loss of one sodium from the neutral compound. Subsequently, each of the major ions had equally intense or less intense ions which differed by 22 amu, corresponding to monoanions of an acidic monoprotonated SBE-β-CD. The $(SBE)_{7b}$-β-CD was included for comparison purposes; nevertheless, the major ion corroborated the degree of substitution of the sample.

As a general observation, the FABMS obtained in the TEA matrix (Fig. 5a) appears to be more sensitive for the higher substituted CDs than those obtained in the GLY matrix. As can be observed from the figure, the complexity of each spectrum increases with the increase in substitution level. The complexity of the spectrum may be indicative not of the purity of the sample, but of the heterogeneity of the mixture of isomers present in the band. Wenz [13] reports an estimation of the possible positional isomers as a function of the number of identical substituents, with three positional isomers for the monosubstituted β-CD increasing to approximately 12,000 for the substitution of eight hydroxyl groups. These numbers are only an approximation, since there are regio isomers and steric factors to be considered, but the numbers support the complication of FABMS spectra interpretation for highly substituted SBEs. Nevertheless, the results obtained did allow for a clear assignment of the ds of each band based in the molecular ions of each sample.

Molar response factors and actual composition of the SBE-β-CD mixtures.—Although the purity of the samples was not thoroughly established, the solids were used to obtain the molar response factor to estimate the composition of a mixture of $(SBE)_{4m}$-β-CD with a ds of 3.6. As can be observed in Table 1, a linear correlation between the analyte response and concentration was obtained in each case and the regression line had an insignificant y-axis intercept (P values > 0.08). The CE molar response obtained

Table 1
Capillary electrophoresis molar response factor (slope) obtained with each band under the following conditions: uncoated capillary, 50 μm i.d., 365 μm o.d., 57 cm total length, 50 cm effective length; field strength, 526 V cm^{-1}; running buffer, 23 mM benzoic acid–25 mM Tris, pH 7.0; indirect detection, UV 230 nm; sample introduction, 5 s at 0.5 psi; temperature of separation, 25 °C

ds	MW	n	Slope	Intercept	R2
0	1134	4	7.71E + 07	−1.96E + 03	0.999
1	1292	5	2.04E + 08	1.08E + 04	0.997
2	1450	5	4.04E + 08	−7.85E + 04	0.989
3	1608	4	5.34E + 08	−1.63E + 05	0.985
4	1768	4	6.87E + 08	−7.49E + 04	0.994
5	1924	5	9.71E + 08	−2.03E + 04	0.997
6	2082	4	1.13E + 09	−5.41E + 04	0.998
8	2398	4	1.75E + 09	−1.71E + 05	0.997

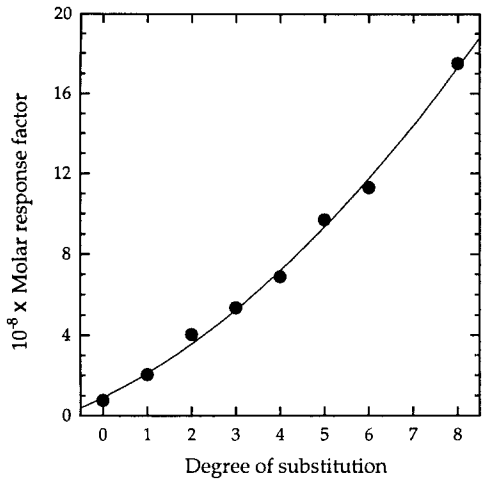


Fig. 6. Dependence of the molar response factors on the degree of substitution of each band.

with each band clearly increases with the increase of the ds of the SBE-β-CD and the correlation between the molar response factors of each band and the ds is shown in Fig. 6. This correlation was found to follow a second-degree polynomial.

In order to estimate the actual mass contribution of the individual bands, a $(SBE)_{4m}$-β-CD with ds_{EA} 3.6 was analyzed under the CE conditions used to obtain the molar response factors. Fig. 7 presents the comparison of the pattern of distribution of the $(SBE)_{4m}$-β-CD calculated based on the percentage total peak area and the pattern of distribution as calculated from the percentage of the molar contribution of the individual bands corrected for the molar response factors. The percentage total peak area, which represents the percent contribution of each peak to the total peak area of a given sample [13], assumes the same response factor for all the components of the mixture. As can be observed from the figure, the actual mass percent distribution of the mixture is not symmetrical but skewed toward the lower degrees of SBE substitution with the maximum of the distribution pattern of percent total peak area distribution between bands with ds 4 and 5, while the maximum shifts to a lower ds (between bands 3 and 4) for the percent total mass distribution. The latter result is closer to the ds_{EA} for this mixture.

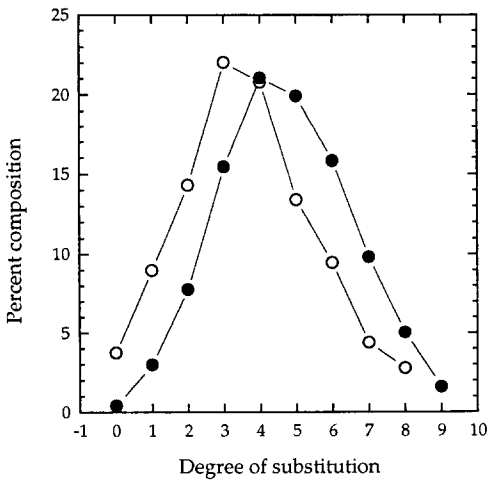


Fig. 7. $(SBE)_{4m}$-β-CD ($ds_{EA} = 3.6$) distribution pattern calculated as; ○ area corrected for mass and; ● total peak area. The run was performed under the capillary electrophoresis conditions of Fig. 3.

In summary, the preparative fractionation of mixtures of SBE-CDs to produce the substitution bands is successfully accomplished by anion-exchange chromatography with sodium sulfate step-gradient elution and pH control. The components of the mixture are well resolved based on the differences in charge. The degree of substitution of each band is clearly assigned by FABMS, and the purity of the samples as compared to the original mixture is established by CE analysis. A better characterization of the $(SBE)_{4m}$-β-CD is accomplished through a mass contribution definition of the composition of the product mixture. The values for the average degree of substitution derived from this method have a better agreement with those calculated from the elemental analysis (ds_{EA}).

3. Experimental

Chemicals.—All chemicals used were of at least analytical reagent grade purity. Benzoic acid and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NaOH, Na_2SO_4, and 1,4-butane sultone were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). β-Cyclodextrin was a gift from American Maize Products (Hammond, IN, USA). Thin-layer chromatography (TLC) Kieselgel 60 F_{254} aluminum precoated plates were obtained from EM Science (Cherry Hill, NJ, USA). The SBE-β-CD mixtures were synthesized according to the general procedure of Stella and Rajewski [1,2,12]. The reproducibility of the preparation is acceptable between batches and chemists [13].

General conditions for capillary electrophoresis analysis.—All solns were prepared in freshly double-distilled water. NaOH (0.1 N) was prepared

and stored in a glass bottle. The running buffer, 30 mM benzoic acid–Tris, pH 6.0, was prepared as previously described [13]. The pH 7.0 running buffer was prepared by dissolving 23.0 mmol of benzoic acid (2.90 g) and 25 mmol (3.03 g) of Tris in water, diluting to volume (1 L), filtering through a 0.22 μm filter to remove particulates, and sonicating for 5 min to complete the degassing.

The CE experiments were conducted on a P/ACE 2210 capillary electrophoresis system (Beckman Instruments, Inc., Fullerton, CA), which used a 365 μm o.d., 50 μm i.d. column (PolyMicro Technologies Inc., Phoenix AZ, USA) with an on-column detection window. Data acquisition was performed on a PC-compatible computer using Gilson 712 acquisition software. New capillaries were conditioned by washing them with 0.1 N NaOH for 10 min at 20 psi, 10 min at 0.5 psi, and rinsing 5 min at 20 psi with water. The washing sequence previous to each injection was as follows: at 20 psi, 2 min 0.1 N NaOH, 1 min water, and 2 min running buffer. The buffer at both ends of the capillary was replaced every three runs. Experimental details of CE analyses are included in the figure captions.

Fractionation of the SBE-β-CD mixture.—The SBE-β-CD mixture was fractionated by AEC, and the experiment was conducted as described below.

(a) Mobile phase preparation. The mobile phase (0.001–1 M Na_2SO_4) was prepared by dissolving the corresponding amount of salt in 1000 mL of freshly double-distilled water. The pH of the solns 0.001–0.5 M Na_2SO_4 was adjusted to 3.5–4.0 with 5% H_2SO_4. The solns were used with no further treatment.

(b) Column preparation. The DEAE Sephadex A-25 (Pharmacia LKB, S-75182, Uppsala, Sweden) was soaked for 2 days at room temperature in 0.5 M Na_2SO_4, pH 3.5–4.0, with occasional stirring, and the supernatant was changed four times with fresh buffer to complete the swelling. The gel was packed as a thick slurry into a glass column. The resin bed was conditioned for 20 h with very slow flow and washed with water until the eluant tested free of sulfate by $BaCl_2$ precipitation (0.5% w/v, pH 2.0).

(c) Experimental fractionation. A soln of a $(SBE)_{4m}$-β-CD (25 g dissolved in 75 mL water), containing from β-CD up to deca-substituted material, was loaded on to the resin. Once all the sample soln was drained into the column, the elution by gravity flow at a constant solvent height of 30 cm was initiated. The mobile phase was a step-gradient from water to 0.5 M Na_2SO_4 at pH 3.5–4.0 and from 0.5–1 M Na_2SO_4 without adjusting the pH. The elution rate was approximately 2 mL/min (reduced at 0.3 mL/min overnight). The fractions were homogenized and checked for the presence of SBE-β-CD, first by spotting a TLC plate, followed by charring after spraying with H_2SO_4–vanillin, and then analyzed by CE without treatment. The different bands were identified by their migration times and their identity was confirmed by spiking the samples with a mixture of known composition of $(SBE)_{4m}$-β-CD. The results are summarized in Fig. 3.

(d) Fraction processing. After the CE identification, the fractions containing the same compound were pooled, neutralized with 0.1 N NaOH to pH 7.0, and concd at 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 ultrafiltered through a cellulose membrane (Diaflo, YC5, 75 mm, AMICON, Inc., Beverly, MA 0915, USA) previously activated with double-distilled water during a 1-h period (replacing the water every 15 min.). The desalting was performed at 15–20 psi and monitored until an aliquot of the filtrate tested negative for sulfate by the addition of 0.5% w/v $BaCl_2$, pH 2.0. The retentate was filtered through a 0.22-μm nylon filter, concd to approximately 100 mL, and lyophilized. The fractions containing β-CD were not processed. The bands with ds 1–4 were dialyzed through a 500 molecular weight cutoff (MWCO) membrane (Diaflo YC5, Amicon). The bands with ds 5 and higher were concd to approximately one-half volume, and the excess of Na_2SO_4 was allowed to precipitate overnight at 5 °C. The precipitate was removed by vacuum filtration, and the remaining soln was dialyzed through a 1000 MWCO membrane (Diaflo YC10, Amicon). The Na_2SO_4 precipitated was analyzed by CE and exhibited some SBE-β-CD. No attempt was made to recover this material. The total mass of SBE-β-CD recovered (CE peaks 1–8) was approximately 60% of the original mixture loaded on to the column with higher yield for the bands with low ds.

Characterization of SBE-β-CD bands.—(a) Capillary electrophoresis analysis. The CE conditions and results are presented in Fig. 4. The sample concn for each band was 1.3–1.6 mg/mL of running buffer. The band corresponding to CE peak number 7 was accidentally contaminated during the workup, and it was included in the figure for comparison purposes only.

(b) Nuclear magnetic resonance. The proton NMR spectra of each band was obtained at ambient temper-

ature (QE-300 NMR spectrometer, General Electric) using a frequency of 300.7 MHz. Standard pulse sequences were employed. The sample concns were 40–60 mg/mL of D_2O.

(c) Mass spectrometry. Mass spectra were obtained on a AUTOSPEC-Q tandem hybrid mass spectrometer (VG Analytical Ltd., Manchester, UK) equipped with an OPUS data system. Fast-atom-bombardment mass spectrometry experiments were performed using a cesium gun operated at 20 keV energy and 2 μA emission. The samples were dissolved in distilled water to a concn of 10–12 μg/μL and added to glycerol or triethanolamine as the matrix. Exact-mass FAB experiments were carried out at 1:10,000 resolution using linear voltage scans under data system control and collecting continuum data mode. Matrix and matrix/potassium adduct (or polyethylene glycol) ions served as bracketing calibrant ions. The results obtained are summarized in Fig. 5.

Composition of SBE-β-CD mixtures.—(a) Molar response factors. The CE conditions and results obtained are summarized in Table 1. Stock solns of 5–6 mg of the pure bands (previously dried under vacuum at 120 °C for 16 h) were prepared in 2-mL volumetric flasks, and additional water dilutions were prepared to provide a concn range between 0.5 and 4 mg/mL. The samples were injected in triplicate. The linear curve fitting was by the least-squares method.

(b) Actual composition of an SBE-β-CD mixture. A sample of $(SBE)_{4m}$-β-CD (25 mg/mL water) was analyzed by CE under the same conditions used for the determination of the molar response factors. The molar response factor for $(SBE)_{7b}$-β-CD was obtained from the correlation between the molar response factor versus the ds of the band and was adjusted with a second-degree polynomial fit (Fig. 6). The molar concn of each substitution band was calculated from the peak area values corrected from the corresponding molar response factor. The percent total molar composition was calculated as the area of the band times 100 divided by the sum of the molar concns of bands with ds 1–8. The values were compared to the percent total peak area composition (percent contribution of each peak to the total peak area of the sample). The results are presented in Fig. 7.

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