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Research Article

Comparative analysis of the full set of methylated β-cyclodextrins as chiral selectors in capillary electrophoresis

The chiral separation ability of the full library of methylated-β-cyclodextrins towards pharmacologically significant racemic drugs including basic compounds was studied by chiral CE. The syntheses of all the methylated, single isomer β-cyclodextrins were revised and optimized and the aqueous solubility of the derivatives was unambiguously established. The three most relevant commercially available methylated isomeric mixtures were also included in the screening, so a total of ten various methylated CDs were investigated. The effects of the selector concentration on the enantiorecognition properties at acidic pH were investigated. Among the dimethylated β-cyclodextrins, the heptakis (2,6-di-*O*-methyl)-β-cyclodextrin isomer (2,6-DIMEB) resulted to be the most versatile chiral selector. Terbutaline was selected as a model compound for the in-depth investigation of host-guest enantiodiscrimination ability. The association constants between the two terbutaline enantiomers and 2,6-DIMEB were determined in order to support that the enantioseparation is driven by differences is host-guest binding. The migration order of the enantiomers was confirmed by performing spiking experiments with the pure enantiomers. 1D and 2D NMR spectroscopy was applied to the 2,3-, and 2,6-DIMEB/terbutaline systems to rationalize at molecular level the different enantioseparation ability of the dimethylated β-cyclodextrin selectors.

Keywords:
Crystalline methylated cyclodextrin / Dimethylated cyclodextrins / Enantioseparation / Randomly methylated cyclodextrin / Single isomer

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Chiral capillary electrophoresis (CCE) has been applied frequently as a simple and reliable analytical technique in mainly pharmaceutical analysis [1–3]. Having its major advantages (such as the high plate numbers, the consumption of minute amounts of aqueous solutions, the straightforward method development in short time, the capability of high throughput, i.e. fast screening setup, and the possibility of the reversal of the migration order, etc.) over the most commonly used LC methods makes CCE very popular in analytical scale enantioseparations. Despite all these, CCE is usually not the first choice in case analysts face enantiomeric purity determination challenges. Unfortunately, CCE methods are rather underrepresented in pharmacopoeial monographs as well. It has been argued that general methods describing the enantioseparation have to be more elaborated in order to add

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Abbreviations: 2,3-DIMEB, heptakis (2,3-di-*O*-methyl)-β-cyclodextrin; **2,6-DIMEB**, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin; **3,6-DIMEB**, heptakis (3,6-di-*O*-methyl)-β-cyclodextrin; **2-MEB**, heptakis (2-*O*-methyl)-β-cyclodextrin; **3-MEB**, heptakis (3-*O*-methyl)-β-cyclodextrin; **6-MEB**, heptakis (6-*O*-methyl)-β-cyclodextrin; **BCD**, β-cyclodextrin; **CCE**, chiral capillary electrophoresis; **CRYSMEB®**, crystalline methylated-β-cyclodextrin; **DIMEB50**, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin ~ 35%; **DMSO**, dimethyl sulfoxide; **DS**, degree of substitution; **HDMCM**, heptakis(2,3-di-*O*-methyl-6-*O*-carboxymethyl); **KOH**, potassium hydroxide; **Pd/C**, palladium on activated charcoal; **PTC**, phase-transfer catalysis; **RAMEB®**, randomly methylated-β-cyclodextrin; **ROESY**, rotating frame nuclear Overhauser effect spectroscopy; **TBAF**, tetrabutylammonium fluoride; **THF**, tetrahydrofuran; **TRIMEB**, heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin)

Color online: See the article online to view Figs. 1–5 in color.

irrefutable data about the paramount importance of CCE in separation science. More than 10 years ago, Holzgrabe presented that CCE has superiority over LC in many of the cases and emphasized that a careful and valid experimental procedure results in a competitive CCE method [4]. In recent years, there is still a growing number of CCE methods published, in which CCE has been supported by NMR and/or molecular modeling to provide a solid background of the enantioseparation at the molecular level.

CDs are water-soluble cyclic oligosaccharides, shaped like a truncated cone, with a central hydrophobic cavity. These sugars have been extensively utilized as excipients in pharmaceutical and food industry [5], and due to their specific 3D arrangement and inherent chirality, they have been used as analytical tools for enantioseparation, especially as chiral selectors (i.e. BGE additives) in CCE [6, 7]. However, the selection of the appropriate chiral selector for CCE is still challenging. It is still not well predictable how the chosen selectors will perform under given conditions using the analyte in question.

Recently, there are countless semisynthetic CD derivatives with various substituents (bearing fit-for purpose functionalities in the introduced sidechains); however, their wide applicability can mostly be rationalized by their ease of availability. The application of native, unmodified CDs has certain advantages being available at a low prize, but among the limitations – especially for that of the most commonly applied β-cyclodextrin (BCD) – one can mention its relatively low aqueous solubility. This drawback hampers its use where higher selector concentrations are necessary for achieving the required selectivity. To overcome the solubility issues, different synthetic methodologies were developed for the chemical derivatization of BCD [8]. Random functionalization processes with methylating agents result in ill-defined mixtures, but these processes are usually up-scalable, making possible the industrial application of these derivatives. Methylated CDs are one of the most effective solubilizers of poorly soluble organic compounds [9–11], but also frequently used as chiral selectors [12–15]. Selectively methylated BCDs, also called single-isomer methyl-CDs derivatives have been prepared through multistep reactions (heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin [TRIMEB]), through repeated chromatographic and crystallization cycles (heptakis (2,6-di-*O*-methyl)-β-cyclodextrin [2,6-DIMEB]) or through exhaustive use of protecting groups (heptakis (3,6-di-*O*-methyl)-β-cyclodextrin [3,6-DIMEB] for example) and applied mainly in separation sciences and in chemosensing. Although methylated CDs are used in diverse applications, their detailed structural characterization is still challenging [16, 17]. The degree and the site of methylation influences the properties such as solubility, complexation ability, and enantiorecognition of the CD, therefore it is of vital importance to gain as complete picture as possible on these features. The enantiorecognition ability of various methylated CDs has already been investigated previously; however, those studies did not cover the full set of the available CDs or did not use single chemical entities but mixtures of variously under-/over-methylated isomers [18–20]. The effect of degree of methylation on the enantiomer migration order has also been shown for peptide enantiomers reviewed by G.K.E Scriba [21]. It has been recently shown, that the degree of methylation can also affect the enantiomer migration order as well [22].

CE in conjunction with NMR spectroscopy can provide valuable information regarding the structure and selector-selectand complexes, therefore appointing the moieties responsible for enantiodiscrimination [23–26]. In our study, all the single isomer per-monomethylated, per-dimethylated, and the commercially available randomly substituted CDs along with the per-trimethylated derivative were screened using a set of racemic compounds in CCE to establish structure-enantiodiscrimination relationships. To get an atomic level picture on the enantiorecognition, a commonly used model drug terbutaline was chosen [12, 27–29].

2 Materials and methods

2.1 Chemicals and materials

The BCD was the product of Wacker Chemie AG (München, Germany); randomly methylated-β-cyclodextrin (RAMEB®; CAVASOL® W7 M), crystalline methylated-β-cyclodextrin (CRYSMEB®), heptakis (2,6-di-*O*-methyl)-β-cyclodextrin ~ 35% (DIMEB50; 2,6-DIMEB content ~ 35%) were products of Wacker, Roquette and CycloLab, respectively. Syntheses solvents such as pyridine (Pyr), tetrahydrofuran (THF), methanol (MeOH), acetone (ACE), *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO) were of reagent grade and were sourced from Molar Chemicals (Halásztelek, Hungary); methyltriphenylphosphonium bromide (98%), benzyl bromide (Bn-Br, 98%), lithium iodide (anhydrous, beads, 10 mesh, 99.99% trace metals basis), lithium hydride (powder, 30 mesh, 95%), (4-(dimethylamino)pyridine (DMAP, ≥99%), tetrabutylammonium fluoride (TBAF, 98%), ammonium hydrogen difluoride (99%), palladium on activated charcoal (Pd/C, 10%), potassium hydroxide (KOH, 85%), *tert*-butyldimethylsilyl chloride (TBDMSi-Cl, 97%), methyl iodide (ReagentPlus®, 99%), hydrazine carbonate (70% in water, ca. 7.3 M), deuterium oxide (D_2O, 99.9% atom % D), chloroform-*d* ($CDCl_3$,99.8 atom % D), dimethyl sulfoxide-d_6(DMSO-d_6, 99.96 atom % D) were sourced from Sigma Aldrich (St. Louis, MO, USA).

Most of the studied racemates such as tapentadol, octopamine, carvedilol, verapamil, methylenedioxipyrovalerone (MDPV), 4-methylethcathinone (4-MEC), flephedrone (4-FMC), mephedrone (4-MMC), butylone (βk-MBDB), vincamine, vincadifformine, vinpocetine, primaquine, tafenoquine, mefloquine, propranolol, bisoprolol, pindolol, atenolol, pantoprazole, ketoconazole, chlorpheniramine, cetirizine, and metoprolol were purchased from commercial suppliers and were of analytical/pharmaceutical grade. Racemic dapoxetine and alogliptin were synthesized as described previously [14, 30]. Terbutaline enantiomers were

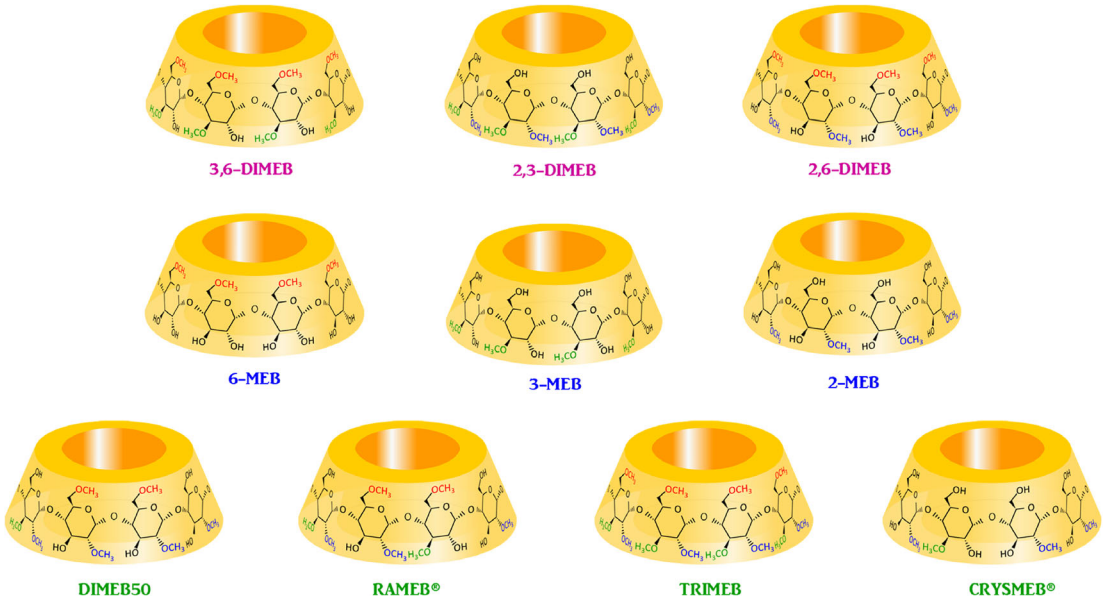

Figure 1. Cartoon representations of methylated β-cyclodextrin derivatives.

isolated and kindly provided by Prof. B. Chankvetadze according to their recent publication [28].

2.2 Syntheses of methylated CDs

Figure 1 shows the cartoon representations of the complete set of methylated β-CDs utilized in this study.

The detailed descriptions of the procedures, the synthetic schemes with atoms-numbered structures and NMR data are shown in the Supporting Information. TRIMEB was prepared by exhaustive methylation of RAMEB® under phase-transfer catalysis (PTC) conditions in THF by using methyl iodide as alkylating agent, KOH as base, and triphenylmethylphosphonium bromide.

Heptakis (2,3-di-*O*-methyl)-β-cyclodextrin (2,3-DIMEB) was prepared in three synthetic steps. Native BCD was regioselectively functionalized on primary hydroxyl groups with TBDMSi-Cl in pyridine, then complete methylation of the secondary side was achieved under phase-transfer catalysis conditions and mild removal of primary-side protecting groups with ammonium bifluoride in methanol yielding the titled compound [31].

2,6-DIMEB was obtained by recrystallizion of DIMEB50 in hot acetone, hot methanol, and by cromatography on silica with acetone as eluent in isocratic elution.

3,6-DIMEB was prepared in five steps according to a variation of the procedure described by Stoddart [32]. The benzylation of heptakis(2,6-di-*O*-*tert*-butyldimethylsilyl)-BCD was accomplished under PTC conditions in THF with KOH as base, benzyl bromide as alkylating agent and triphenylmethylphosphonium bromide as catalyst. The conversion to heptakis(2-*O*-benzyl-3,6-di-*O*-*tert*-butyldimethylsilyl)-BCD is exhaustive at room temperature and does not require purification by chromatography. Removal of silyl groups was achieved in THF at room temperature with TBAF while methylation was performed under PTC conditions in THF with KOH as base, methyl iodide as alkylating agent, and triphenylmethylphosphonium bromide as catalyst. The final cleavage of the benzyl groups was obtained by hydrazine-mediated transfer-hydrogenation.

Heptakis (2-*O*-methyl)-β-cyclodextrin (2-MEB) was prepared according to a variation of the procedure described by Stoddart [32]. The methylation of heptakis(2,6-di-*O*-*tert*-butyldimethylsilyl)-BCD was accomplished under PTC conditions in THF with KOH as base, methyl iodide as alkylating agent, and triphenylmethylphosphonium bromide as catalyst. The conversion to heptakis(2-*O*-methyl-3,6-di-*O*-*tert*-butyldimethylsilyl)-BCD is exhaustive at room temperature and does not require purification by chromatography. The final removal of silyl groups was achieved in THF at room temperature with TBAF.

Heptakis (3-*O*-methyl)-β-cyclodextrin (3-MEB) was prepared in a five-step synthesis. The primary side of the BCD was protected with TBDMSi-Cl in pyridine. Regioselective per-2-*O*-benzylation was achieved in DMSO with lithium hydride, benzyl bromide, and lithium iodide as catalyst. Heptakis (2-*O*-benzyl-6-*O*-methyl)-BCD was obtained after purification by chromatography with hexane: EtOAc 9:1 as eluent in isocratic elution. Exhaustive 3-*O*-methylation was achieved under PTC conditions (THF as solvent, potassium hydroxide as base, methyl-iodide as alkylating agent, and triphenylmethylphosphonium bromide as catalyst). Deprotection of the primary-side was accomplished with TBAF at room temperature overnight in THF. Debenzylation of the secondary-side was attained by applying hydrazine-carbonate in the presence of Pd/C.

Heptakis (6-*O*-methyl)-β-cyclodextrin (6-MEB) was prepared according to a five-step synthesis. The primary hydroxyl groups of the native BCD were selectively protected

with TBDMSi-Cl in pyridine. Benzylation of the secondary side was achieved by PTC (THF was used as solvent, KOH as base, benzyl bromide as alkylating agent and triphenylmethylphosphonium bromide as catalyst) under mild conditions without the need of rigorous dry environment. The reaction crude was purified by simple precipitation with excellent yields. Deprotection of the primary-side (*O*-desilylation) was exhaustively accomplished with excess of (harmless) TBAF at room temperature overnight in THF. Per-6-*O*-methylation of the CD ring was also achieved by PTC with methyl iodide. The removal of benzyl moieties, used as temporary secondary-side protecting groups, was attained by applying hydrazine-carbonate in the presence of Pd/C.

2.3 Instrumentations and methods

CE measurements were carried out on an Agilent 7100 instrument (Agilent Technologies, Waldbronn, Germany), equipped with a photodiode array detector (DAD) and the Chemstation software for data handling. Measurements were performed in untreated fused silica capillaries (33.5 cm total and 25 cm effective length and 50 μm id) purchased from Agilent Technologies. Prior to all runs, the capillary was preconditioned by rinsing with 0.1 M NaOH (2 min), water (2 min), and the appropriate BGE (3 min). The temperature of the capillary was set to 20°C. During measurements 20 kV was applied, UV detection was performed at 200 nm. Samples were injected hydrodynamically (40 mbar × 3 sec). The running buffer was 20 mM phosphoric acid (85%) adjusted to pH 2.5 with 1 M NaOH. The BGE contained the appropriate methylated-BCDs at 1, 2.5, and 5 mM concentrations for the less soluble 2-MEB and 6-MEB, and 10, 20, and 30 mM for 2,3-DIMEB, 3,6-DIMEB, 2,6-DIMEB, DIMEB50, CRYSMEB®, RAMEB®, and TRIMEB. Stock solutions of the investigated analytes were prepared at 1 mg/mL concentration in methanol and their 50-fold dilution with water was used to prepare working solutions for CE analysis.

The enantioresolution (R_S) values were calculated with the formula:

$$R_S = \frac{2\ (t_r - t_s)}{w_r + w_s} \tag{1}$$

where t_r and t_s are the migration times of the enantiomers and w_r and w_s stand for the extrapolated peak widths at the baseline.

For the determination of enantiomer migration order in case of terbutaline, MeOH stock solutions of *S*-(+)-terbutaline (1 mg/mL) and *R*-(-)-terbutaline (1 mg/mL) were prepared. *R*-(-)-terbutaline stock solution was diluted with water 50-fold, while *S*-(+)-terbutaline stock solution was diluted 25-fold with water to obtain work solutions. These work solutions were mixed to obtain the work solution 'terbutaline spiked' in which the concentration of *S*-(+)-terbutaline was doubled with respect to *R*-(-)-terbutaline.

For the determination of association constants, the racemic terbutaline sample was prepared by 50-fold dilution of the methanol stock solution with water containing 0.1 v/v% DMSO serving as EOF marker. The experiment was carried out by running the sample in BGEs containing increasing concentration of 2,6-DIMEB (0–10–15–20–25–30–35–40 mM). The basic principle of this set up is the assumption that the presence of the CD selector affects the migration velocity of the guest in case the complexation takes place (Note: in the current system, the selector is neutral, while the guest is positively charged, therefore increasing concentration of the host results in decreasing the mobility of the guests, thereby upon complexation terbutaline enantiomers migrate slower). Obviously, according to changes in the BGE (e.g. increase in viscosity due to 2,6-DIMEB addition), the migration time of the neutral species may change as well. By relating the migration time of the guests to that of the EOF, these effects are compensated during the calculations. In general, the more stable complex is formed with the host, the more migration time of the free guest is influenced. From the dataset of migration times and selector concentrations, the stability constant can be determined with the x-reciprocal method [15, 23, 33, 34]. Although this is a simple and still routinely used method in the case of fast screening experiments, evaluations with non-linear fittings provide more reliable data and also complex mobility values. The effective electrophoretic mobility (μ_{eff}) can be obtained from:

$$\mu_{eff} = \frac{l_c l_d}{U} \cdot \left(\frac{1}{t} - \frac{1}{t_0}\right) \tag{2}$$

where l_c is the total length of the capillary, l_d is the length of the capillary to the detector, U is the applied voltage, while t and t_0 are the peak appearance times of the analyte and the EOF marker, respectively.

To obtain the cyclodextrin-terbutalin binding constants, the experimental μ_{eff} versus cCD dataset has to be fitted by the following function:

$$\mu_{eff} = \frac{\mu_{free} + \mu_{cplx} K\,[CD]}{1 + K\,[CD]} \tag{3}$$

where μ_{free} is the mobility of terbutaline in the absence of CD, μ_{cplx} and K are the electrophoretic mobility and the binding constant of the terbutaline–CD complex, respectively.

All NMR experiments were carried out on a 600 MHz Varian NMR spectrometer (using a DirectDigital Receiver) equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. For structural characterization of the synthesis intermediates and final CD products, standard pulse sequences and processing routines available in VnmrJ 3.2 C/Chempack 5.1 were used. The complete resonance assignments were established from direct ^{1}H–^{13}C, long-range ^{1}H–^{13}C, and scalar spin–spin connectivities derived from 1D ^{1}H, ^{13}C, ^{1}H–^{1}H gCOSY, ^{1}H–^{13}C gHSQCAD, ^{1}H–^{13}C gHMBCAD experiments, respectively. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The ^{1}H chemical shifts were referenced to the applied NMR solvent in each case. The detailed NMR study of racemic terbutaline and 2,6-DIMEB was performed in D_2O under acidic conditions. Appropriate amount of racemic terbutaline HCl and 2,6-DIMEB were dissolved to obtain a 1:1.5 molar ratio

(2:3 mM). The individual resonances of terbutaline were assigned by spiking the solution with single enantiomer terbutaline. Spatial proximities were deduced from two-dimensional rotating frame nuclear Overhauser effect spectroscopy (2D ROESY) experiment (using a mixing time value of 300 msec) with standard experimental setup used in Chempack.

3 Results and discussion

3.1 Enantioseparation by CE

The three representatives of the methylated mixture of isomers, RAMEB®, CRYSMEB®, and DIMEB50 are materials with rather different compositions and properties. RAMEB® is a mixture of randomly methylated isomers with an average degree of substitution (DS) around 12 with high aqueous solubility (see Supporting Information Table 1 for the data about the aqueous solubilities of all the methylated CDs). The pattern of substitution of the isomeric populations is almost statistical (the three *O*-methyl signals in the ^{1}H-NMR spectrum corresponding to 2-*O*-, 3-*O*-, and 6-*O*-substitution are 1:1:1). CRYSMEB® is a mixture of methylated isomer with low DS (around 4) and the isomers composing the material are mainly (if not exclusively) substituted on the secondary side (i.e., 2-*O*- and 3-*O*-substituted). The material is more soluble in water than native BCD, but its aqueous solubility is not pronounced. DIMEB50 is a mixture of (di)methylated isomers with a DS of 15–16. The main component of the mixture is the 2,6-DIMEB (around 35% based on our *in-house* CycloLab HPLC method) and the material has a very high aqueous solubility.

Among the three selected randomly methylated composite products and under the tested experimental conditions, the best performing methylated mixture of isomers was DIMEB50. The product first prepared in industrial scale by Chinoin (Hungary) allowed to separate 12 out of the 27 pairs of screened racemates. RAMEB® and CRYSMEB® performed efficiently as versatile chiral selectors as well, taken into accounts that ten and nine pairs of stereoisomers were successfully separated, respectively. In more detail, tapentadol, carvedilol, flephedrone, vinpocetine, mefloquine, pantoprazole, and terbutaline were at least partially separated by all the three methylated composite materials (see Table 1 for all the R_s values).

DIMEB50 performed best with flephedrone (R_s = 1.18 at 3.3 mM), mefloquine ($R_{s\,diast}$ = 4.69 at 10 mM), and terbutaline (R_s = 2.16 at 10 mM), RAMEB® was superior in resolving tapentadol ($R_{s\,diast}$ = 4.32 at 10 mM) and vinpocetine ($R_{s\,diast}$ = 3.4 at 30 mM, see Fig. 2), while CRYSMEB® could effectively separate carvedilol (R_s = 1.13 at 10 mM). Pantoprazole was equally resolved by all the three methylated composite materials (R_s = 0.46 at 10 mM).

Among the random methylated materials, DIMEB50 was the only selector able to provide partial resolution for mephedrone (R_s = 0.42 at 20 mM) and butylone (R_s = 0.4 at 10 mM), CRYSMEB® uniquely allowed separation for MDPV (R_s = 0.35 at 10 mM), while RAMEB® permitted partial seperation of propranolol (R_s = 0.21 at 10 mM). Octopamine and pindolol were partially resolved by RAMEB® (R_s = 0.6 and R_s = 0.4 at 30 mM, respectively), DIMEB50 (R_s = 0.91 and R_s = 0.43 at 30 mM, respectively), 2,6-DIMEB (R_s = 0.88 and R_s = 0.43 at 30 mM), while CRYSMEB® was ineffective for these racemic mixtures, a high DS of methylation seems necessary for the enantioseparation of these drugs. The case of ketoconazole is more difficult to rationalize: enantioseparation was achieved effectively with DIMEB50 (R_s = 1.67 at 30 mM) and CRYSMEB® (R_s = 1.6 at 30 mM), while RAMEB® was an inefficient additive in this case. The enantiomers of this antifungal medication could also be resolved by using TRIMEB (which provided the best resolution among all tested methylated derivatives, R_s = 3.17 at 30 mM) and 2,6-DIMEB (R_s = 1.44 at 20 mM); it can be argued that for the enantioseparation of racemic ketoconazole, the methylation at position O(2) is necessary but not sufficient (2,3-DIMEB and 2-MEB were ineffective).

The prepared methylated single isomers have remarkable differences in their solubility and the unique case of 3,6-DIMEB is worth mentioning. The negligible aqueous solubility of this compound did not allow its screening as chiral selector under the applied aqueous experimental conditions. This property of 3,6-DIMEB challenges all the previous reports claiming the enantiodiscrimination ability of 3,6-DIMEB under aqueous conditions. In most cases, when authors refer in papers to 3,6-DIMEB as the applied chiral selector, it is in fact a mixture of isomers [18, 20]. Among the per-dimethylated single isomers, 2,6-DIMEB has the highest aqueous solubility, 2,3-DIMEB possesses intermediate solubility, while 3,6-DIMEB is very slightly soluble in water. The per-monomethylated derivatives, 2-MEB, 3-MEB, and 6-MEB, have generally lower aqueous solubility compared to the per-dimethylated compounds (in some case, almost one order of magnitude less). 3-MEB has the highest solubility in water, while 2-MEB and 6-MEB possess remarkably lower solubility.

All the methylated single isomers (and the random composite materials as well) were capable of providing separation for the stereoisomers (diastereomers and/or enantiomers) of tapentadol to some extent. The set-ups including TRIMEB ($R_{s\,diast}$ = 10.82, R_{s2} = 0.93 at 30 mM) and 2,3-DIMEB ($R_{s\,diast}$ = 11.72, $R_{s\,2}$ = 1.25 at 30 mM) were performing particularly efficiently as they could provide simultaneous separation of one diastereomeric pair ($R_{s\,diast}$) and the later migrating pair of enantiomers (R_{s2}). From these results it is clear that for this opioid drug high DS and a fully methylated secondary side seems ideal, while methylation on primary side is of less importance. This observation is in agreement with our previously published results on the remarkable enantiorecognition ability of heptakis(2,3-di-*O*-methyl-6-*O*-carboxymethyl)-BCD (HDMCM) towards tapentadol [35]. HDMCM was able to baseline separate both enantiomeric pairs at high pH values which was attributed to the electrostatic interactions between the negatively charged guests and the positively charged hosts. Surprisingly, at low pH

Table 1. Enantioresolution values obtained by using various methylated β-cyclodextrins

	RAMEB			CRYSMEB			DIMEB50			TRIMEB			2,3-DIMEB			2,6-DIMEB			2-MEB			3-MEB			6-MEB		
	10 mM	20 mM	30 mM	10 mM	20 mM	30 mM	10 mM	20 mM	30 mM	10 mM	20 mM	30 mM	10 mM	20 mM	30 mM	10 mM	20 mM	30 mM	1 mM	2.5 mM	5 mM	10 mM	20 mM	30 mM	1 mM	2.5 mM	5 mM
Tapentadol	4.32	3.16	2.14	2.99	1.92	1.4	1.97	1.08	0.90	6.77	9.79	10.82	7.46	9.6	11.72	1.55	0.5	0	6.05	4.8	3.27	1.21	2.3	4.29	5.8	5.1	3.8
									0.25	0.74	0.99	0.93	0.97	1.12	1.25				0.42				0.8	1.71	0.5		
Dapoxetine	0	0	0	0	0	0	0	0	0	2.02	1.94	1.68	1.35	1.73	1.96	0	0	0	0	0	0	0.61	0.9	1.03	0	0	0
Octopamine	0	0.56	0.6	0	0	0	0.43	0.79	0.91	0	0	0	0	0	0	0.54	0.69	0.88	0	0	0	0	0	0	0	0	0
Carvedilol	0.95	0.64	0.4	1.13	0.81	0.55	0.83	0.41	0	0	0	0	0	0	0	0.62	0	0	1.12	1.42	1.37	0	0	0	0	0.49	0.60
Verapamil	0	0	0	0	0	0	0	0	0	1.15	1.85	2.95	0	0	0.22	0	0	0	0	0	0	0	0	0	0	0	0
Methylenedioxypyrovalerone	0	0	0	0.35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.24	0	0	0	0.52	0.80	1.46	*
4-Methylethcathinone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Flephedrone	0	0	0.53	0	0	0.38	0.8	1.1	1.18	0	0	0	0	0	0	1.09	1.32	1.43	0	0	0.51	0	0	0	0	0	0
Mephedrone	0	0	0	0	0	0	0.36	0.42	0.42	0	0	0	0	0	0	0.5	0.49	0.52	0	0	0	0	0	0	0	0	0
Butylone	0	0	0	0	0	0	0.4	0.23	0	0	0.52	0.82	0	0	0.17	0.41	0.18	0	0	0.31	0.43	0	0	0	0	0	0
Vincamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vincadifformine	0	0.00	0.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vinpocetine	3.07	3.38	3.40	1.44	1.71	1.79	0.77	0.74	0.68	0	0	0	0	0	0	0	0	0	0	0	0.32	0	0	1.15	0	0	0
Primaquine	0	0	0	0	0	0		0	0	0	0	0.81	0	0	0	0.56	0.64	0.75	0	0	0	0	0	0	0	0	0
Tafenoquine	0	0	0	0	0			0	0	0.42	0.53	0.53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mefloquine	2.83	2.79	2.44	2.25	2.93	3.26	4.69	3.55	2.88	0	0	0	0	0	0.27	6.1	4.9	4.18	1.16	2.08	3.96	0	0	0.54	0	0	0
Propranolol	0.21	0	0	0	0	0	0	0	0	0	0	0.31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bisoprolol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pindolol	0	0.29	0.4	0	0	0	0	0.39	0.43	0	0	0	0	0	0	0.2	0.4	0.43	0	0	0	0	0	0	0	0	0
Atenolol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.19	0.38	0	0	0	0	0	0	0	0	0
Pantoprazole	0.46	0	0	0.47	0	0	0.47	0	0	0	0	0	0	0	0	0	0	0	0.71	1.03	1.04	0	0	0	0	0	0
Ketoconazole	0	0	0	0.76	1.2	1.6	1.02	1.57	1.67	1.71	2.59	3.17	0	0	0	1.04	1.44	1.72	0	0	0	0	0	0	0	0	0
Chlorpheniramine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cetirizine	0	0	0	0	0	0	0	0	0	0	0	0	1.02	1.69	2.42	0	0	0	0	0	0	0	0	0	0	0	0
Terbutaline	1.98	2.08	2.07	1.65	1.66	1.56	2.16	1.89	1.67	0	0	0.55	0	0	0	1.97	1.68	1.6	0.86	1.31	1.73	0	0	0	0	0.60	1.36
Metoprolol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alogliptin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2,3-DIMEB, heptakis (2,3-di-*O*-methyl)-β-cyclodextrin; 2,6-DIMEB, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin; 2-MEB, heptakis (2-*O*-methyl)-β-cyclodextrin; 3-MEB, heptakis (3-*O*-methyl)-β-cyclodextrin; 6-MEB, heptakis (6-*O*-methyl)-β-cyclodextrin; CRYSMEB®, crystalline methylated-β-cyclodextrin; DIMEB50, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin ~ 35%; RAMEB®, randomly methylated-β-cyclodextrin; TRIMEB, heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin.

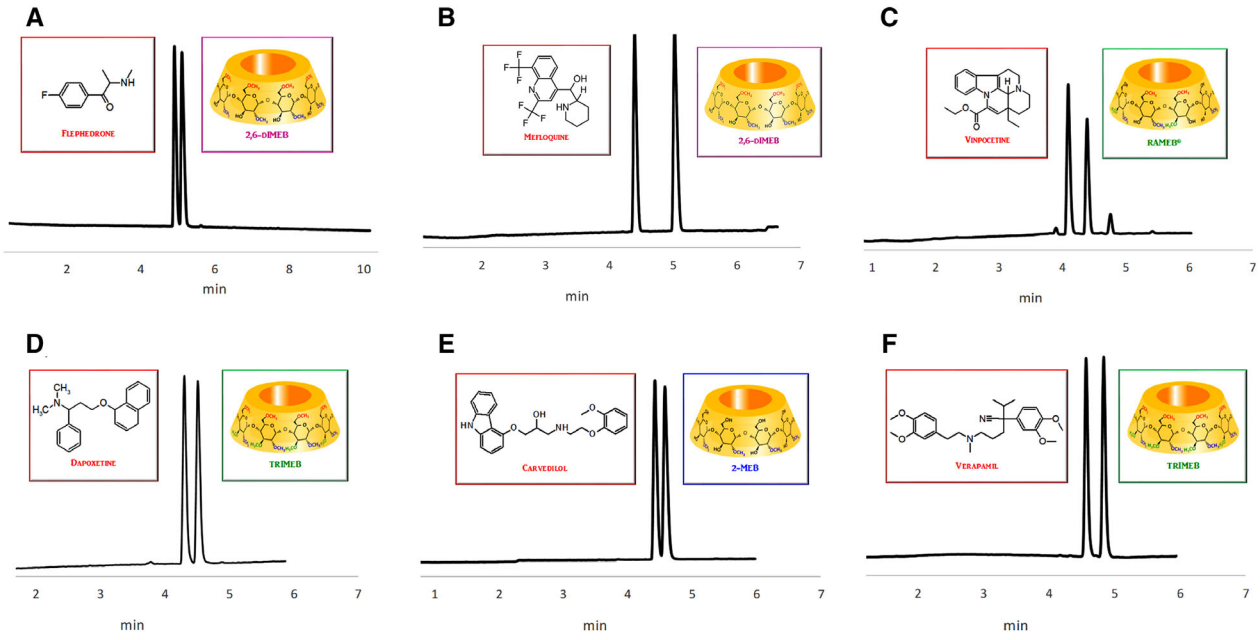

Figure 2. Representative CE electropherograms obtained with various methylated β-cyclodextrins: (A) 30 mM 2,6-DIMEB, (B) 2.5 mM 2,6-DIMEB, (C) 30 mM RAMEB®, (D) 30 mM TRIMEB, (E) 10 mM 2-MEB, (F) 30 mM TRIMEB (33.5 cm total and 25 cm effective length and 50 μm id capillary, 20 mM H_3PO_4-NaOH pH 2.5, 20 kV; 20°C, 200 nm).

value ionic interactions are less predominant, HDMCM was still able to resolve the later eluting (RS/SR) enantiomeric pair. Under the light of these results and by taking into consideration the structural similarities between HDMCM and 2,3-DIMEB (both are completely substituted on the secondary side) it can be argued that, in principle, the 2,3-dimethylated BCD scaffold is the key factor which governs tapentadol enantioresolution. An analogue scenario was obtained for the dapoxetine enantiomers. In this case, the use of a BCD exclusively methylated on the O(3) positions (3-MEB) already allowed a satisfactory enantioseparation ($R_s = 0.9$ at 20 mM); however, the utilization of the analogue exhaustively methylated on the secondary side (2,3-DIMEB) improved remarkably the enantiodiscrimination ($R_s = 1.96$ at 30 mM) while the application of the fully methylated counterpart (TRIMEB) only slightly improved the separation ($R_s = 2.02$ at 10 mM). For the separation of the dapoxetine enantiomers, the simultaneous and exhaustive methylation of the secondary rim has a synergistic effect, while methylation on the primary side has a minor influence. Carvedilol was effectively separated by both methylated composite materials (all the three tested) and methylated single isomers. The adrenergic receptor blocker racemate was best separated by 2-MEB (see Fig. 2E); however, some resolution was achieved with 6-MEB ($R_s = 0.60$ at 5 mM) and 2,6-DIMEB ($R_s = 0.62$ at 10 mM) as well. Selective methylation on the O(3) positions seems detrimental for the discrimination of this pair of enantiomers, as 3-MEB, 2,3-DIMEB, and TRIMEB were completely ineffective.

The methylated BCD selectively substituted on the primary position, 6-MEB was particularly effective for the separation of MDPV ($R_s = 1.46$ at 2 mM), 2-MEB and 3-MEB showed minor performances ($R_s = 0.24$ at 2.5 mM and $R_s = 0.52$ at 30 mM, respectively), while the remaining tested CD derivatives (with the exception of CRYSMEB®) were ineffective. It seems that a low DS is favorable for the enantioseparation of this recreational drug.

Flephedrone and mephedrone racemates were best separated by 2,6-DIMEB (see Fig. 2A) and among the remaining single methylated isomers only 2-MEB exerted some recognition ability towards flephedrone ($R_s = 0.51$ at 2.5 mM). Simultaneous and exhaustive 2,6-methylation seems ideal for the separation of these synthetic stimulant drugs and for the separation of primaquine. The antimalarial drug was only effectively resolved by applying 2,6-DIMEB ($R_s = 0.75$ at 30 mM) or TRIMEB ($R_s = 0.81$ at 30 mM). On the other side, the exhaustive primary side methylation seems unfavorable for the separation of the alternative antimalarial drug, mefloquine as TRIMEB and 6-MEB were the only derivatives not showing enantiorecognition ability.

Selective methylation on the O(2) seems an important feature for the effective separation of butylone. TRIMEB (best performing, $R_s = 0.82$ at 30 mM), 2,6-DIMEB ($R_s = 0.41$ at 10 mM), 2,3-DIMEB ($R_s = 0.17$ at 30 mM), and 2-MEB ($R_s = 0.43$ at 5 mM) were effective for partially resolving the enantiomers of the psychoactive drug.

Single methylated isomers were not effective selectors for vinpocetine and in this case, the composite methylated materials outperformed them. However, 2-MEB and 3-MEB were showing some separation ability ($R_s = 0.32$ at 5 mM and $R_s = 1.15$ at 30 mM, respectively). Propranolol was best separated by the fully methylated TRIMEB ($R_s = 0.31$ at 30 mM), while pantoprazole was successfully resolved by 2-MEB ($R_s = 1.04$ at 5 mM). For the enantioseparation of terbutaline, exhaustive substitution on the O(3) seems unfavorable.

The racemic mixtures of verapamil and tafenoquine were uniquely separated by the fully methylated TRIMEB (see Fig. 2F), cetirizine racemate was only and successfully resolved by applying 2,3-DIMEB, while the enantiomers of atenolol were effectively separated only by using the 2,6-DIMEB as the chiral selector. Metoprolol, bisoprolol, vincadifformine, vincamine, 4-methylethcathinone, chlorpheniramine, and alogliptin were not separated under the tested experimental conditions by any of the methylated BCD derivatives.

To summarize, methylated CDs are effective tools for chiral separations of different variety of compounds. The enantiorecognition ability of a chiral selector is difficult to predict and in most of the cases challenging to rationalize. The methylated isomeric mixtures are, in general, more versatile chiral selectors. Taking into account that these derivatives are easier to prepare and commercially available at a reasonable price, they could be suggested as first-choice methylated selectors. However, 2,6-DIMEB showed exceptional enantiorecognition abilities among the single isomers and in general among the methylated derivatives. The fact that this derivative is a single component is undoubtedly advantageous in terms of batch to batch reproducibility, impurity profile setting, and revealing the detailed mechanism of separation by NMR spectroscopy to provide a comprehensive theoretical framework for the underlying mechanisms of enantiomeric separation. On the other side, the time-consuming synthetic steps for the 2,6-DIMEB preparation make this compound less appealing. At this regard, TRIMEB (or even 2-MEB) could be suggested as valuable alternative. These two single isomers showed good versatility as chiral selectors, and their preparation and scale-up were achieved effectively.

3.2 CE characterization of the terbutaline-2,6-DIMEB host-guest system: Binding constant and enantiomer migration order determinations

The migration order of the terbutaline enantiomers in the 2,6-DIMEB containing BGE was determined by injecting terbutaline 'spiked' working solution into the electrophoretic system. Electropherogram in Supporting Information Fig. 1 shows that the *R*-(-)-terbutaline is the first migrating component while *S*-(+)-terbutaline migrates slower, meaning that the migration velocity of *S*-(+)-terbutaline is more affected by 2,6-DIMEB, also revealing that this isomer has somewhat higher affinity towards 2,6-DIMEB (Supporting Information Fig. 1A).

Based on this observation, another CE experiment was performed aiming the quantification of the association constants between 2,6-DIMEB and the two terbutaline enantiomers. 2,6-DIMEB was added at various concentrations (0–1–5–10–15–20–35–40 mM) to the BGE (Supporting Information Fig. 1B). The increasing concentrations of the selector resulted in altered migration times of the peaks of both terbutaline enantiomers. The association constants were determined by non-linear fitting and were found to be 225 ± 24 for the first migrating *R*-(-)-terbutaline (Supporting Information Fig. 2A) and 318 ± 43 for the second migrating *S*-(+)-terbutaline (Supporting Information Fig. 2B). The complex mobility values were found to be the same for both enantiomers (Supporting Information Fig. 2).

3.3 Structural characterization of the terbutaline-2,6-DIMEB host-guest system by NMR

In order to get a deeper insight into the molecular interactions between the 2,6-DIMEB and terbutaline enantiomers, ^{1}H and 2D ROESY NMR experiments were performed according to previous works [3, 31]. The enantioselectivity of 2,6-DIMEB was monitored at acidic pH with racemic terbutaline. In the ^{1}H NMR spectrum of the racemic terbutaline and the 2,6-DIMEB host, complexation induced chemical shift changes could be observed for all the non-exchangeable protons of terbutaline (see Fig. 3).

Spiking the terbutaline-2,6-DIMEB system with enantiopure *R*-(-)-terbutaline, the ^{1}H NMR resonances of the enantiomers could be assigned based on resonance intensity differences (see Supporting Information Fig. 3).

2D ROESY NMR spectrum was recorded in order to further support the observed interactions between 2,6-DIMEB and terbutaline at the atomic level. A partial ROESY spectrum of the terbutaline: 2,6-DIMEB system is shown in Fig. 4. Intense cross-peaks can be observed between the aromatic moiety of terbutaline and the inner cavity protons of 2,6-DIMEB, suggesting that the dihydroxyphenyl ring is fully immersed into the cavity.

It is worth mentioning that under the same experimental conditions, this part of the guest molecule does not interact with the methylated analogue 2,3-DIMEB as clearly shown by the absence of cross-peaks in the 2D ROESY spectrum of this system (see Supporting Information Fig. 4 for the ROESY spectrum). As the resonances of the aromatic moiety show intense cross-peaks exclusively with the inner 2,6-DIMEB protons H3, H5, and with the protons of the methyl groups located on the primary rim (CD-(O)6-CH_3) and taken into consideration that the resonances of the tertbutyl moiety of the guest (protons H10, H11 and H12) show intense cross-peaks with the protons of the methyl groups located on the secondary rim (CD-(O)2-CH_3) (see Supporting Information Figs. 5 and 6 for the full ROESY spectrum and the ROESY enlargement of the methyl protons, respectively), an inclusion arrangement in which the phenyl ring is located at the proximity of the CD primary interacting with the surrounding methoxy groups can be hypothesized. A model for the inclusion complex of terbutaline and 2,6-DIMEB is schematically shown in Fig. 5 using key ROESY interactions for the determination of terbutaline orientation in the cavity.

The arrangement may also be favoured by the polar interactions between the positively charged secondary amine and the secondary OH groups of the host.

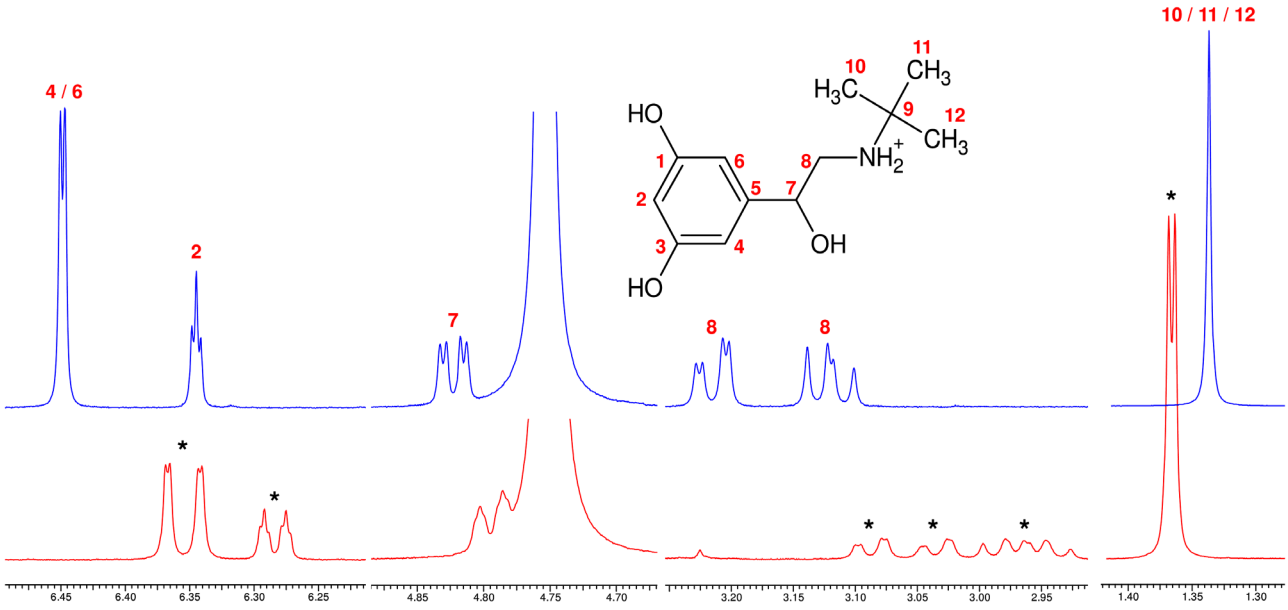

Figure 3. The ^{1}H NMR spectrum of racemic terbutaline (blue colour, top) and the ^{1}H NMR spectrum of racemic terbutaline: 2,6-DIMEB solution at 1:1.5 molar ratio (red, bottom). The latter shows remarkable enantioresolution effects as indicated by *.

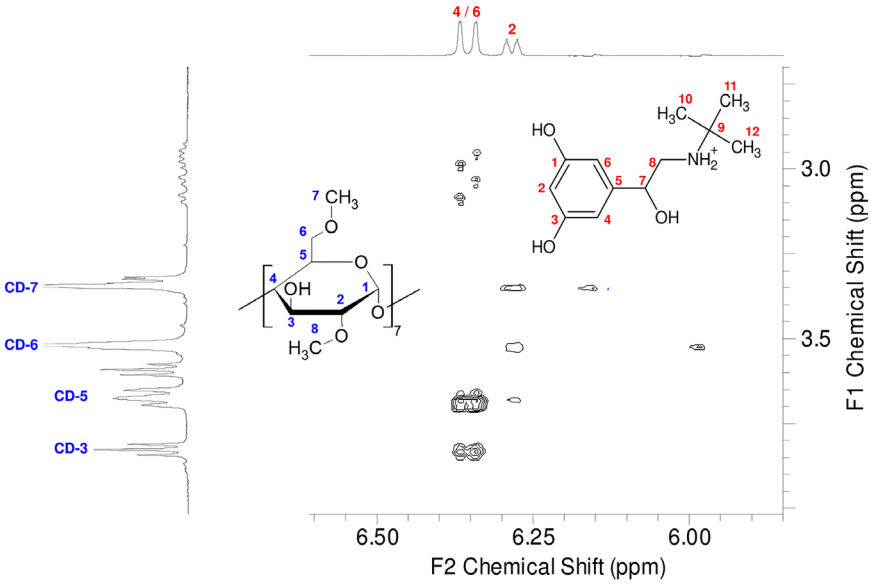

Figure 4. Partial 2D ROESY NMR spectrum of racemic terbutaline and 2,6-DIMEB showing intense cross-peaks between the inner CD protons (H3, H5) and aromatic protons of terbutaline.

4 Concluding remarks

Herein, we have reported the updated and optimized preparations of all the methylated single isomer CDs using current synthetic methodology. Among all developed procedures, the synthesis of TRIMEB was found to be the most straightforward: the one step reaction starting from RAMEB® provided an easy scale-up and this material can be produced without difficulties in kg scale. The syntheses of the DIMEB and the various MEB derivatives require multiple steps and extensive use of protecting group, and as a consequence the industrial scale-up is challenging. However, the application of PTC conditions to each alkylating step allowed the production of the methylated derivatives in multi-gram scale (10–100 g scale). The complete set of single isomers mono-, di-, and trimethylated derivatives supplemented with the commercially available randomly substituted analogues were subjected to a screening experiment in capillary electrophoresis as chiral selectors. 3,6-DIMEB could not be included in the study due to aqueous solubility issues. We have concluded that the isomeric mixtures are in general more versatile chiral selectors. Among the dimethylated ones, 2,6-DIMEB was the more versatile in enantioseparation. Terbutaline was used as a model guest to highlight the role of methylation pattern in enantiorecognition. 2D ROESY NMR experiments confirmed that 2-*O*- and 6-*O*-methylation extends the cavity

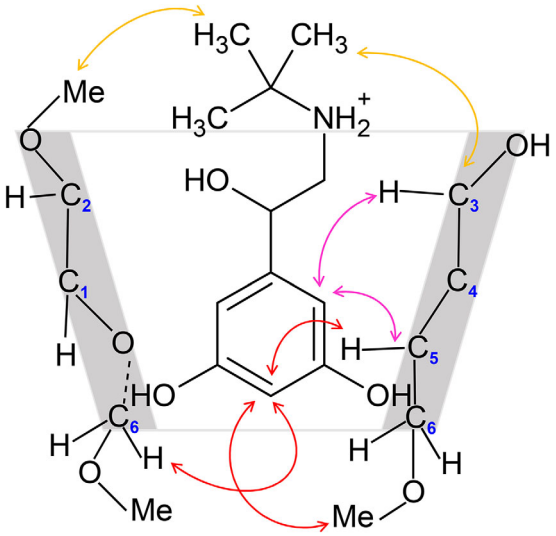

Figure 5. Proposed geometric arrangement of terbutaline in the cavity of 2,6-DIMEB, based on the ROESY experiment.

to accommodate terbutaline in an enantiospecific manner. As an alternative to the single isomer, 2,6-DIMEB, TRIMEB, or 2-MEB could also provide similar advantages.

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The authors have declared no conflict of interest.

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