THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Biocompatibility evaluation and synthesis of macrocyclic compounds

by Eszter Róka

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SUPERVISORS:
Dr. Ildikó Bácskay
Dr. Florent Perret

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DEBRECEN, 2017

Contents

Abbreviations

^{13}C NMR	^{13}C NMR carbon-13 nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
AcACD	acetylated-α-cyclodextrin
ACD	α-cyclodextrin
ADI	acceptable daily intake
API	active pharmaceutical ingredient
AT	antithrombin
BAM	biologically active molecule
BCD	β-cyclodextrin
BCS	biopharmaceutical classification system
Bn	benzyl
BnBr	benzyl bromide
BSE	bovine spongiform encephalopathy
Bu	butyl
BuBr	butyl bromide
C4S	*para*-sulphonato-calix[4]arene
C6S	*para*-sulphonato-calix[6]arene
C8S	*para*-sulphonato-calix[8]arene
CD	cyclodextrin
$CDCl_3$	deuterated chloroform
CE	complexation efficiency
CGTase	cyclodextrin glucanotransferase
$CHCl_3$	chloroform
CI	cell index
CMACD	carboxymethylated-α-cyclodextrin
CMACDEp	carboxymethylated-α-cyclodextrin crosslinked with epichlorohydrin
CMBCD	carboxymethylated-β-cyclodextrin
CMC	critical micelle concentration
CnS	*para*-sulphonato-calix[n]arene
COSY	correlation spectroscopy
D_2O	deuterium oxide
DEPT	distortionless enhancement by polarization transfer

DHPBCD	(2,3-dihydroxypropyl)-β-CD
DIMEA	hexakis (2,3-di-O-methyl)-α-cyclodextrin
DIMEB	heptakis (2,3-di-O-methyl)-β-cyclodextrin
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DS	degree of substitution
EI	electrical impedance
EM	exact mass
ESI	electron spray ionisation
Et	ethyl
EtI	ethyl iodide
Eur. Pharm.	European Pharmacopoeia
FAO/WHO	Food and Agriculture Organization of the United Nations and World Health Organization
FDA	Food and Drug Administration
GI	gastrointestinal tract
HBSS	Hank's balanced salt solution
HC II	heparin cofactor II
HC_{50}	concentration which causes 50% hemolysis
HDMBr	hexadimethrine bromide
HPACD	(2-hydroxypropyl)-α-cyclodextrin
HPBCD	(2-hydroxypropyl)-β-cyclodextrin
HPGCD	(2-hydroxypropyl)-γ-cyclodextrin
HPLC	high pressure liquid chromatography
HPMC	hydroxypropyl methylcellulose
IC_{50}	half maximal inhibitory concentration
INS	international numbering system for food additives
iv.	intravenous
J	coupling constant
$K_{1:1}$	stability constant
K_d	dissociation constant
LDH	lactate dehydrogenase
LY	Lucifer Yellow
m-	meta-

MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
Me	methyl
MeI	methyl iodide
MeOD	deuterated methanol
MS	mass spectrometry
MS	molecular substitution
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	molecular weight cut-off
NaCMC	carboxymethylcellulose sodium salt
NaH	sodium hydride
NMR	nuclear magnetic resonance
o-	ortho-
p-	para-
PARMEA	partially methylated α-cyclodextrin
PARMEB	partially methylated β-cyclodextrin
PBS	phosphate-buffered saline
PEG	polyethylene glycol
Pgp	p-glycoprotein
PI	phosphatidylinositol
Pr	propyl
PrI	propyl iodide
PrP^{C}	prion protein
PrP^{SC}	pathogenic prion protein
PS	average number of substituents
PUFA	poly-unsaturated fatty acid
RAMEA	random methylated α-cyclodextrin
RAMEB	random methylated β-cyclodextrin
RBC	red blood cell
RS	ring substitution
RT-CES	Real Time Cell Electronic Sensing
S	substituent group/unit
S_0	intrinsic solubility
SBEBCD	sulfobutylether-β-cyclodextrin
SuACD	succinylated-α-cyclodextrin

$t_{1/2}$	half life time
TBAF	tetra-n-butylammounium fluoride
TC_{50}	half maximal toxic concentration
TEER	transepithelial electrical resistance
THF	tetrahydrofuran
TMSCl	trimethylsilyl chloride
TRIMEA	hexakis (2,3,6-tri-O-methyl)-α-cyclodextrin
TRIMEB	heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin
USP/NF	United States Pharmacopeia and National Formulary
V_d	volume distribution
VRAC	volume-regulated anionic channel
δ	chemical shift

Introduction

Regarding the up-to-date databases of novel drug candidates, the tendency of increased lipophilic nature and molecular size is conspicuous. However, the biological activity properties and solubility are inversely proportional. [1] The Biopharmaceutical Classification System classifies the APIs based on their aqueous solubility and gastrointestinal permeability, as fundamental characteristics. [2, 3] (Figure 1) An orally administered drug has to be dissolved in the aqueous environment along the gastrointestinal tract, however a somewhat lipophilic characteristic is also indispensable for permeating through the membrane barriers.

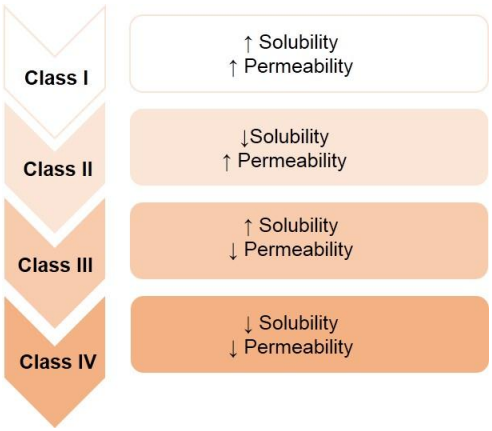


Figure 1 *Biopharmaceutical Classification System*

Macrocyclic compounds consist of repeating units with no terminal ends. Owing to the cyclic structure each possess a cavity, which is suitable for accommodating guest molecules. Highly water soluble macrocyclic rings are able to form inclusion complexes with BCS II and IV APIs, facilitating their solubility, thus bioavailability enhancement.

The major classes of macrocyclic organic host compounds are the cyclodextrins, calixarenes, crown ethers, and cucurbiturils. [4] Cyclodextrins had been known first, and the subsequent extensive investigations resulted their current application not only as pharmaceutical excipients, but as orphan drug as well.

Despite of the practically unlimited possibilities of CD modification, just a few derivatives have been applied in medical formulations, and mainly β-CDs. The biocompatibility of several other β-CDs has been evaluated for more than a decade in the Department of Pharmaceutical Technology, University of Debrecen. [5-7] The small dimension of the α-CD cavity limits the range of guest molecules, which explains their less frequent pharmaceutical application. Our aim was to determine the biocompatibility of those α-CD derivatives, which bear similar

structural modifications than the already studied β-CDs. The biocompatibility evaluation included *in vitro* methodologies, such as cell viability assays and hemolysis test. The apprehension of the correlations between the structural modifications and the toxic effects in each system, helps to ascertain the connection between the chemical structure and the safety profile.

Our commitment towards α-CDs had been reinforced by the research group of Applied Supramolecular Chemistry (CSAp), University of Lyon 1. They have demonstrated an advantage of α-CD application, namely that they form stable nanoparticles, which facilitates sustained drug release. [8-10] The goal of our cooperation with the CSAp group was the synthesis of alkyl ether α-CDs, with well-defined structure. Modifications at the primary, secondary and both faces aimed to clarify how the reducing free hydroxyl group number or the increasing length of the alkyl substituent effects the cytotoxic or hemolytic activity.

In correspondence with the proportion of experimental work performed, the main part of the dissertation deals with cyclodextrins, both in the Bibliographic and Experimental part. The second part of our work was focused on *para*-sulphonato-calix[n]arenes, which have several similarities to CDs. *Para*-sulphonato-calix[n]arenes have been widely studied and have a great possibility to future pharmaceutical application. However, their biocompatibility evaluations are still incomplete. Our aim was to complement the missing data on their effects on Caco-2 cells, more precisely their impact on paracellular absorption from the small intestine.

To sum up, in our work, the focus of attention was on to enrich the already existing data on the structure-activity correlations of cyclodextrins and calixarenes. The experimental work followed the scheme, illustrated on Figure 2. The beneficial characteristics of macrocycles carries practically limitless possibilities in pharmaceutical formulations. Although, the countless number of derivatives requires most the understanding of basic connections between structure and effect, to gain the maximal benefit from their diverse structure, and to use the safest choice in each pharmaceutical formulation.

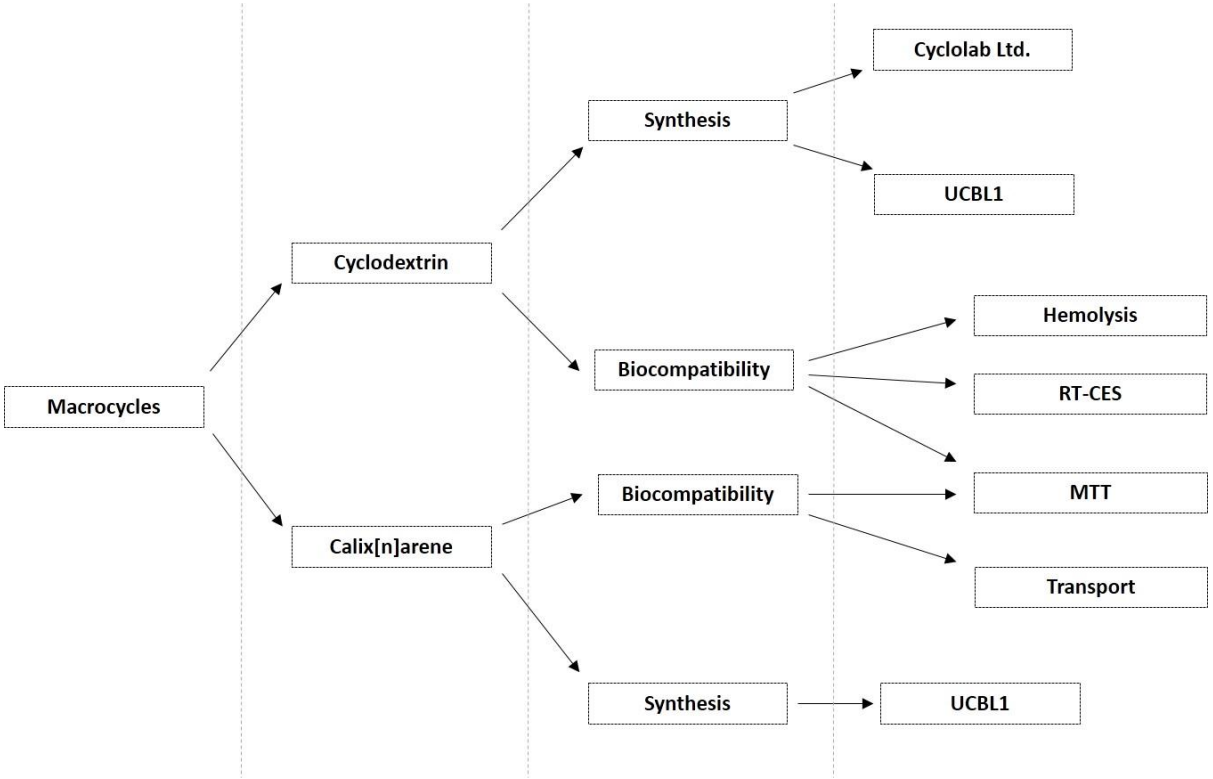


Figure 2 *Scheme of work. UCBL1: Université Claude Bernard Lyon 1.*

Cyclodextrins

I. History

The history of cyclodextrins reaches back until the second half of the 19th century. This last 120 years can be divided into 5 periods based on the classification of József Szejtli [11]:

Period of discovery (1891-1911)

The discovery of CDs started, when Antoine Villiers noted the formation of unwanted crystals during the fermentation of potato starch with *Bacillus amylobacter*. [12-15] (Figure 3)

Franz Schardinger (the Founding Father of Cyclodextrins) discovered the *Bacillus macerans* strain which produced *crystalline dextrin A* and *B* from starch. [16] Until the 1970's, CDs were called as *Schardinger dextrins*.

CHIMIE ORGANIQUE. — *Sur la fermentation de la fécule par l'action du ferment butyrique*. Note de M. **A. Villiers**.

« J'ai montré dernièrement (*Comptes rendus*, février 1891, p. 435) que la fécule de pomme de terre peut, dans des conditions déterminées, fermenter sous l'action du *Bacillus amylobacter*, les produits principaux de cette fermentation étant constitués par des dextrines.

» Il se forme en même temps, mais en très petite quantité, soit environ 3gr pour 1000 de fécule, un hydrate de carbone qui se sépare en beaux cristaux radiés, au bout de quelques semaines, dans l'alcool ayant servi à la précipitation des dextrines. Ces cristaux renferment de l'eau et de l'alcool de cristallisation, la proportion de ce dernier étant très faible, environ 4 pour 100. Au contact de l'air, ils deviennent opaques, en perdant de l'alcool et absorbant de l'eau, sans que leur poids varie d'une manière notable. En les dissolvant dans une assez grande quantité d'eau chaude, on obtient, par refroidissement, de petits cristaux brillants, inaltérables à l'air, dont la composition est représentée par un multiple de la formule

$$C^{12}H^{10}O^{10} + 3HO.$$

Figure 3 *One fragment from the second publication of Antoine Villiers from 1891. [13]*

The period of doubt (1911-1935)

The conflicting ideas between laboratories, and the confusion caused by the unclear terminology resulted in misleading conclusions.

Schardinger dextrins were called *polyamyloses* by Hans Pringsheim [17], who could not conclude the cyclic structure of dextrins, but he was convinced that the glucose units are bonded by α-1,4-glucosidic linkages. [18] Pringsheim performed *in vivo* tests with dextrins, and suggested α-dextrin application in diabetes treatment, as it did not cause any nausea and it did

not increase the urine's sugar level. [19] He studied the complex formation of dextrins and their acetate derivatives with various organic compounds. [20]

Paul Karrer examined first the interactions between dextrins and different cations [21], and the first tosylated derivatives were synthetized by Karl Freudenberg. [22]

Period of maturity (1935-1950)

The development of dextrins started to bloom owing to the successful contributions between laboratories. The first, who suggested the cyclic structure was Karl Johann Freudenberg [23, 24]. The successful separation of γ-dextrins is also bonded to him, and he also proposed the existence of dextrins built up from 9 and 10 units. [17] The final description of the exact weight and the number of building units was achieved by Dexter French, who used a more appropriate nomenclature: *cyclohexaamylose* for α-, *cycloheptaamylose* for β-, and *cyclooctaamylose* for γ-dextrin. [25] The cell-free isolation of the *cycloamylose glucanotransferase* had been successfully carried out by Evelyn B. Tilden, Claude S. Hudson and Karl Myrbäck. [26-30] At the end of the 1940's Cramer was the first who used the *cyclodextrin* name. [31]

Exploration (1950-1970)

French's reported the structure and the dimensions of δ-, ε-, ζ- and η-CDs built up from 9, 10, 11 and 12 glucose units, respectively. [32] He also studied the CD-iodine complexes [33] and the high temperature cellulose column chromatography. [32] However, he made a significant error regarding the toxicity of CDs. [34] In his experiment, β-CD was administered to rats. Based on his observations, the rats refused the CD diet even in small doses, and the experimental animals died without exception within one week. However, neither the number of tested animals, the presence of control groups, nor the information about the purity of the used β-CD were clarified. French stated that CDs present certain toxicity, and he doubted Pringsheim's proposition for using them as potential antidiabetic treatment by calling it 'risky'. This misinformation deterred many scientist and delayed the development of CD containing formulations for pharmaceutical use. French's findings were explained later by the remaining solvent traces in the sample, and as the rats have well developed sense of smell they refused to eat it. [11, 35]

Friedrich Cramer suggested the truncated cone shape of CDs, he reported the relative hydrophobicity of the inner cavity [36], and he studied the driving forces of inclusion complex formation. [37] The first patent concerning the application of CDs was filed in 1953 by Cramer, Freudenberg and Plieninger. [38]

The first ^{1}H NMR characterisation of the CD architecture was finally performed by Benito Casu [39], and by 1979 the industrial scale production of pure CDs became possible.

Period of application (from 1970 until now)

The name of Wolfram Saenger [40-43] and József Szejtli are especially outstanding in this period.

Due to the efforts of the Hungarian researcher József Szejtli, CDs became widely used and their industrial relevance is sharply increased. Szejtli is considered as the Godfather of Cyclodextrins. [17] He had the most important contribution to the industrial application of CDs, notably he founded the Cyclolab Ltd. (in Budapest, Hungary) in 1972. The institute is dedicated to target the technological transfer between CD research and industry. [44] Szejtli approached the inclusion phenomenon from a new aspect. [45] He reported his findings in numerous reviews and books, and his lifetime work has been established the wide-scale usage of CDs, and resulted their sharply increased industrial relevance. [11, 17, 45-48]

In the early 1970s the routine NMR analysis become accessible. The most relevant representatives of NMR characterization are Takeo, Kuge, Demarco and Thakkar. [49]

Firstly, CDs were used as solubilizing excipients for the replacement of co-solvents, organic solvents and surfactants. Later they were used for increasing the stability, bioavailability and several further physicochemical properties of drug molecules. [50] The first CD containing pharmaceutical was marketed in Japan in 1976 (Prostarmon sublingual tablet, with prostaglandin E2). In Europe, the first product appeared in 1988 (Brexin with piroxicam), but in the U.S., only in 1997 (itraconazole oral solution). [51] From the 21th century, some CD derivatives have become issued as orphan drugs, which huge leap confirms their inexhaustible potential.

From the 1980's, the synthesis of CD derivatives had become also a subject of interest of many scientists. Szejtli estimated the number of studied CD derivatives to 1500 in 2004, and since then this number has just continued to grow. Compared to this enormous number, only a few derivatives are used in the pharmaceutical industry, and several of them have not been studied yet *in vitro*. Our aim was to extend the *in vitro* evaluation of α-CDs, for the better understanding of structure-toxicity connections.

II. Chemistry

A. Natural production

The enzyme, responsible for the conversion of amylose is called cycloamylose glucanotransferase (CGTase or cyclodextrin glucanotransferase). It is produced by a variety of bacteria (mostly the members of the *Bacillus* genus), such as aerobic mesophilic species (*Bacillus macerans*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus ohbensis*, *Klebsiella pneumoniae*, *Klabsiella oxytoca*, *Micrococcus luteus*), aerobic thermophilic (*Bacillus stearothermipihulus)*, anaerobic thermophilic (*Thermoanaerobacterium thermosulfurigen*), aerobic alkalophilic (*Bacillus circulans)* or aerobic halophilic bacteria (*Bacillus halophilus)*. [52] Among these, the *B. macerans* and alkalophilic bacilli are the most extensively studied.

The CGTase is the member of α-amylase family of glycosyl hydrolates, thus it is capable of converting starch and related substances (amylose, other polysaccharides) into CDs. It is a multifunctional enzyme, which catalyses three type of transglycosilation reactions: cyclization (intramolecular), coupling and disproportional reaction (intermolecular), furthermore it also has weak hydrolysing activity. [53] (Figure 4) During cyclization, CGTase converts 1,4-linked α-glycans to cycloamyloses. Notedly, the reducing end sugar is transferred to another sugar residue of the same oligosaccharide chain, forming a cyclic compound. In case of CDs, the terminal 4-OH group is the acceptor. The process is reversible, thus the CD ring can be opened by the same enzyme.

Most bacterial CGTases produce mainly α-, β- and γ-CDs, though their function is not limited to the production of these three molecules. In small amount, higher molecular weight CDs were also identified. [32]. CGTases from different bacterial sources diverge in the yield and proportion of the final products [52], and their activity is also effected by the environmental conditions (pH, temperature, time). [54]

The starting point of the industrial scale CD production was when the biotechnological advances led to an improvement in CGTase production. Genetic engineering facilitated the access to enzymes with increased activity, selectivity and specificity towards different CDs. These technological innovations resulted the availability of purified CDs, which were suitable for pharmaceutical application. [17, 51]

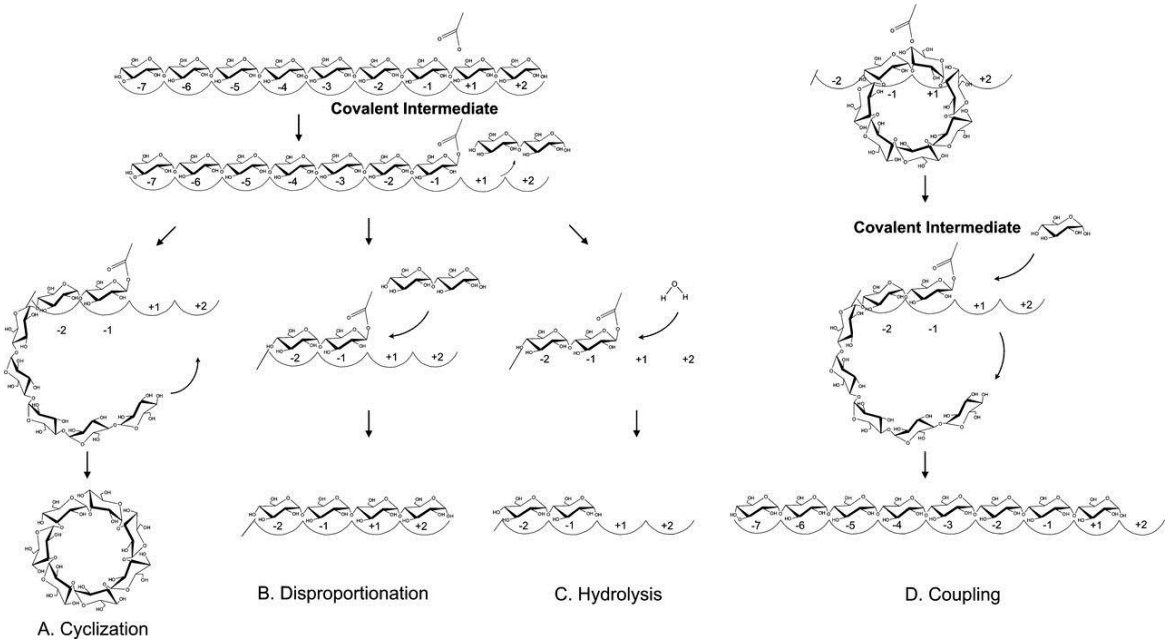


Figure 4 *The four actions of cyclodextrin glucanotransferase: cyclization, coupling, disproportionation and hydrolysis. Figure is adapted from [53].*

B. Structure

CDs are macromolecules built up from D(+) glucopyranose units, which are bound together by α-(1→4) glycosidic linkage. The three major classes of CDs are called α-, β- and γ-CD, consisted of 6, 7 and 8 building units, respectively. (Figure 5) CDs with greater dimension are also described, namely the δ-, ε-, ζ-, η-CDs (9, 10, 11 and 12 units, respectively). [32] Furthermore, the existence of large CDs, even over 100 glucose units was also reported. [55] However, their industrial scale production is rather problematic, due to the particular conditions and the difficulties in purification.

Each glucopyranose ring has one primary hydroxyl group at the C-6 position and two secondary at the C-2 and C-3 positons. The C-1 and C-4 carbons form the glycosidic linkage. Due to the chair conformation of the glucopyranose units, the shape of the CD ring is rather like a truncated cone, than a cylinder. [36] This unique structure requires the special arrangement of functional groups. As a consequence of the C-1 conformation, all hydroxyl groups are positioned on the exterior of the cone. Namely, the primary functions are orientated to the narrow edge of the cone, and the secondary groups are to the wider. The cavity of the torus is lined by two rings of C-H groups, and a ring formed by the ethereal oxygens of the glucose residues. The nonbonding electron pairs of the glycosidic-oxygen is orientated to the inside of the cavity, which position reflects in mild Lewis-base character. [48] Due to this

disposition, the exterior of the cone with the presence of hydroxyl groups is hydrophilic, meanwhile the inner surface of the CD cavity is less hydrophilic. In fact, the aqueous solubility of native CDs is much lower than their linear equivalents due to the C-2 and C-3 positioned hydroxyl groups, which can form intramolecular hydrogen bonds. In case of β-CD, a complete secondary belt is formed, which is an explanation for the rigid structure and low aqueous solubility. In the α-CD ring, one glucose residue by the six is rotated, thus the hydrogen bond belt is not complete. [42] The non-coplanar γ-CD ring has a more flexible structure, which explains its better water solubility. At the narrow end of the CD cone, where the primary hydroxyl groups are situated, the diameter of the cavity entrance is reduced, due to the free rotation of the hydroxyls.

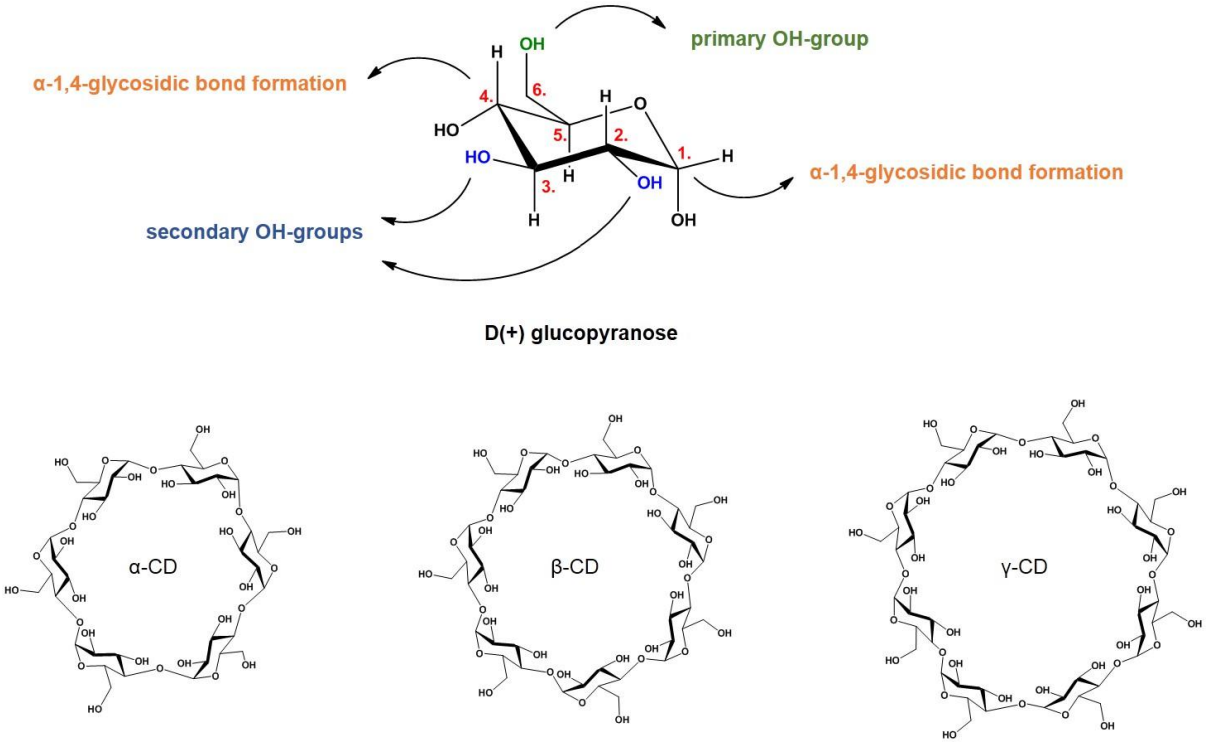
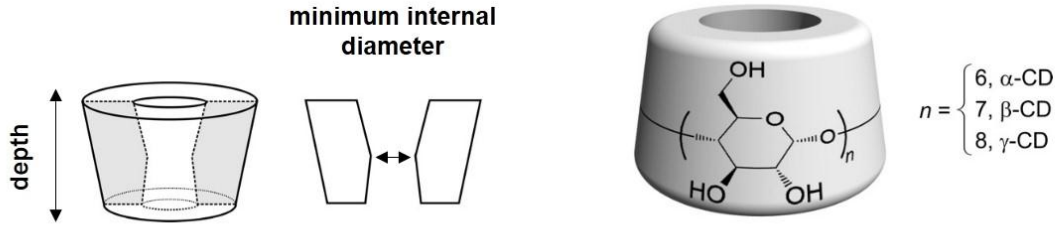


Figure 5 *Structure of a single D(+) glucopyranose molecule and the α-, β- and γ-cyclodextrins.*

Thorough calculations and visualisation methods shed light on the particular CD cavity proportions. [56, 57] The position of H-5 atoms result a definite protrusion of the surface towards the center of the cavity (bottleneck phenomenon). (Table 1) It requires two diameters for the exact characterisation of the CD cavity: an internal and external diameter value. Evidently, the formation of axial inclusion complexes is mainly limited by the internal diameter, thus a suitable guest molecule shall possess a smaller diameter than the internal minimal diameter of the CD cavity.

Table 1 *3D structure, dimensions and characteristics of α-, β- and γ-CD. [48] Figure is modified from [57].*



	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight	972.86	1135.01	1297.15
Water solubility (g/L)	145	18.5	232
Internal minimal diameter (Å)	4.4	5.8	7.4
External diameter (Å)	13.7	15.3	16.9
Cavity volume (Å^3)	174	262	427
Depth (Å)	6.7	7.0	7.0
Approx. cavity volume in 1mol CD (ml)	104	157	256
Crystal forms (from water)	hexagonal plates	monocyclic paralelograms	quadratic prisms
Crystal water (wt%)	10.2	13.2-14.5	8.13-17.7

C. Nomenclature

The lack of unified and clear nomenclature had complicated the progress of CD research for a long time. The name '*cyclodextrin'* has been used since the 1940's, and the abbreviation 'CD' is tolerated in the scientific language. Even though *cyclodextrin* turned to be a general name for the cyclic α-D-(1→4)-linked D-glucose oligosaccharides, it does not contain information on the nature of the intersaccharidic linkages. [55] A systematic nomenclature was proposed where CDs are named '*by giving the systematic name of the glycosyl residue, preceded by the linkage type in parentheses, preceded in turn by 'cyclo' with a multiplicative suffix (i.e. cyclohexakis)'.* In this system α-CD is correctly named as *cyclohexakis-(1→4)-α-D-glycosyl*. [55]

Derivatives with the same substitution pattern on each glucopyranose units are named by adding a multiplicative prefix (e.g. hexakis) to the substituent prefixes, as a group. [58] One confusion is that while mono-substitution refers to one single substituent per CD ring, di- and

tri-substitution means two or three substituents per glucopyranose residue. [59] For instance, DIMEA assigns hexakis(2,3-di-O-methyl)-α-CD, which means the presence of two methyl groups per residue and not two per CD ring.

The unclear interpretation of the substitution degree also led to confusions. For instance, alkylated derivatives were characterized by the following numbers, in the early times: [59]

- 'S' expressed the number of substituted groups per glucopyranose unit, thus the value was between 1 and 3,
- 'DS' meant the average number of substituted hydroxyls per unit, with a number between 0 and 3,
- 'MS' was the *molecular substitution* value, which could be more than three when reactive side chains (e.g. hydroxyalkyl) were present, which could react further,
- 'RS', thus the *ring substitution* gave the number of substituted hydroxyls per CD ring.
- 'PS' marked the *average number of substitution*.

This 'system' evidentially caused several misunderstandings, or in the worst case, they did not have any meaning to the public. In the recent times, DS is conceptually used, and it indicates the number of substituents per CD ring. The *substitution pattern* (SP) term might also be seen sometimes, which refers to the location of the substituent on the glucopyranose unit (or on the side chain), e.g. O-2,-3 or -6. [60]

The DS value can be determined by different analytical methods such as NMR, MS or classical analytical methods (e.g. titration). [61, 62] The following equation is used when the DS is calculated by MS spectra: [63]

$$\mathrm{DS} = \frac{\sum_i I_i \, x \, DS_i}{\sum_i I_i}$$

Where I_i corresponds to the intensity of the i peak, and DS_i is the corresponding DS of the current peak.

D. Derivatives

However, native CDs become available in great quantities for an affordable price, several scientists have been interested in their chemical modification from the 1970's. CD derivatives are synthetized for a variety of reasons and the applied chemical strategy depends on the purpose of the final product. The most demanding principles are: [48, 60, 64]

- solubility modification for aqueous or non-aqueous use,
- extending physicochemical properties (better fit and association with the guest molecule),
- avoiding the interactions between the CD molecules in solution,
- traceability (isotope or fluorescent tagging),
- formation of (in)soluble CD-polymers for reaching a cooperative action of cavities linked together,
- investigation of the mechanism of enzyme-catalysed reactions,
- extending the cavity height,
- intellectual property reason, etc.

All chemical modifications take place at the hydroxyl groups, thus the possible modification pattern is practically limitless. [11, 45]

The first factor, what needs to be considered, is that all hydroxyl groups are nucleophilic in nature, thus the initial step is always an electrophilic attack. The slightly dissimilar reactivity of the primary and secondary hydroxyl groups is auspicious. (Figure 6) Moreover, C-2 and C-3 hydroxyls show further difference in their reactivity, namely those at C-3 position are less reactive, due to their inaccessible position. Primary hydroxyls are the most basic, thus most nucleophilic, while C-2 hydroxyls are the most acidic ones. [42, 65] According to these differences, a weak electrophilic reagent (e.g. TBDMSCl) attacks the primary hydroxyl groups first [66], but it has to be considered that a more reactive reagent (e.g. TMSCl) will indiscriminately react with the secondary hydroxyls, too. [67] As C-2 groups are the most acidic ones, they will be deprotonated first. The formed oxyanion is more nucleophilic than the non-deprotonated hydroxyls at C-6 position. [68] Due to the complicated proton transfers between the two positions, the crude product is usually a mixture of C-2 and C-6 modified derivatives. [69]

The second highly considerable factor is the CD's ability to complex the reagent. [70] If the complex is strong, then the formation of the predominant product is controlled by the orientation of the reagent within the complex. If the complex is weak, then the driving force is the nucleophilicity of the hydroxyl groups. The strength and orientation of the formed reagent-CD complex depend also on the solvent. For instance, α-CD forms a 6-tosylated product with tosyl chloride in pyridine, but in aqueous base the 2-tosylated product forms. Furthermore, the nature of the complex depends on the size of the CD ring: tosyl chloride in aqueous

environment gives the 2-substituted product with α-CD, but with β-CD it gives the 6-tosylated product. [71]

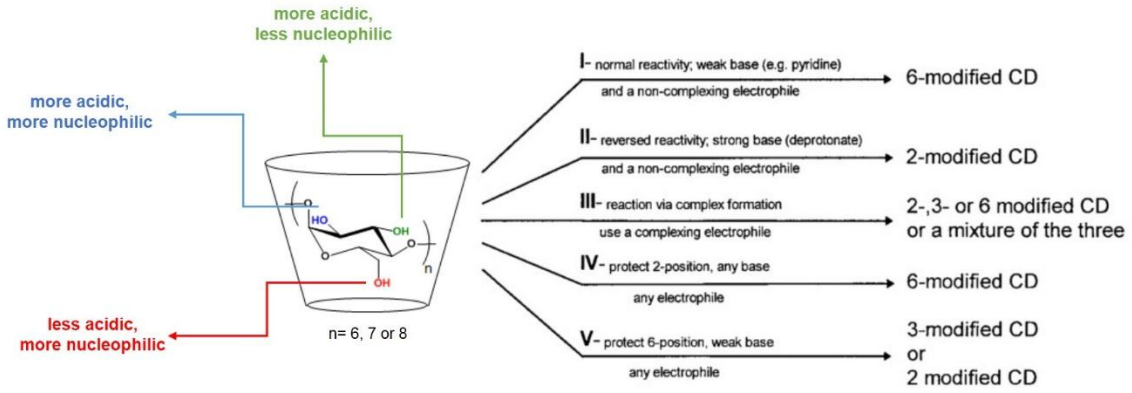


Figure 6 *Selective modification of the primary and secondary hydroxyl groups, based on their different chemical reactivity. Figure is modified from [69].*

The methods for the selective modification can be divided into 3 categories: [69]

1. The '*clever'* method, where the required product is achieved on the shortest possible route. However, this way is only possible when the chemistry of the particular product is well exploited.
2. The '*long'* method when several steps of protection and deprotection lead to the desired compound.
3. The '*sledgehammer'* method where the starting material is discriminately reacted, which eventuate a mixture of products. The desired derivative is separated by chromatographic methods.

The fact, that the final product in CD modification is always a mixture, has to be accepted. Exception can be made with mono- and per-substitutions. Firstly, due to the slight differences between the reactivity of the hydroxyl groups, it is inevitable to have some unwanted under- or over-substituted by-products. Secondly, most of the time, the purification and separation procedure of the crude product is highly elaborate. In practice, the usage of randomly substituted derivatives is common. Therefore, the reinterpretation of the concept 'purity' is desired. In terms of CDs, purity means: [60]

- no residual reagents (alkyl halides, propanesultone, etc. which can be carcinogenic),
- no residual solvents (except the pharmaceutically tolerable ones),

- the residual unsubstituted CD (native parent molecule) level should be as low as possible.

Two type of substitutional processes are distinguished: the regioselective and random modifications. (Figure 7) In case of regioselective substitution, the aim is to modify individually the primary *or* the secondary face (including different ways for C-2 or C-3 hydroxyls). Mono-, di- and tri-substitution refers to the modification of one, two or three hydroxyl groups per glucopyranose residue, respectively. In case of per-substitution, all hydroxyls are modified.

Regioisomers only differ in the relative position of substituents (e.g. the three regioisomers of 6,6-di-substituted α-CD are 6A6B, 6A6C and 6A6D). Some statistical calculations suggest, that di-substitution produce 33 types of regioisomers in case of β-CD. Regiosomerism complicates more the process of derivative formation, although their importance is mainly acknowledged in chromatographic separation and enzymology. [72-74]

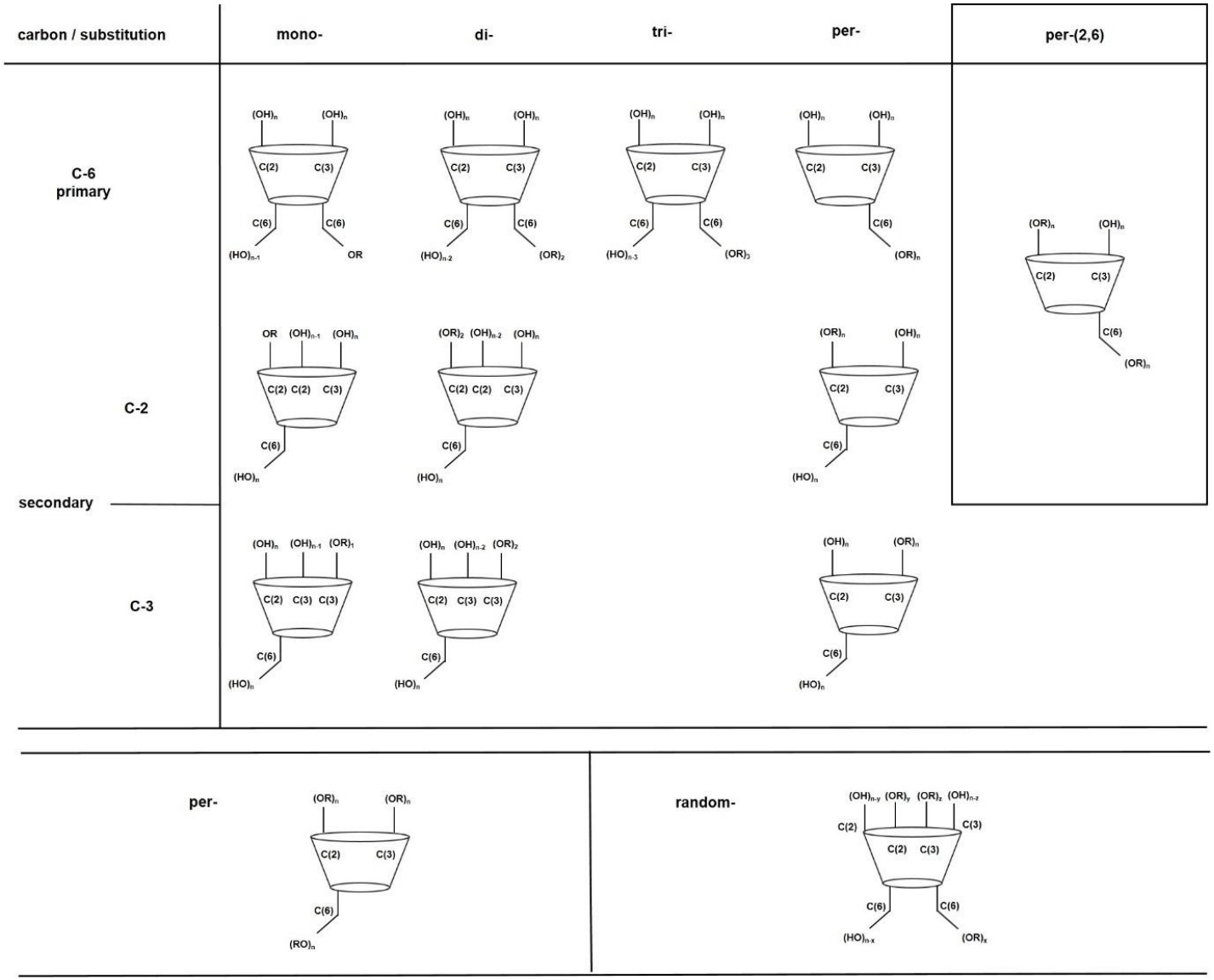
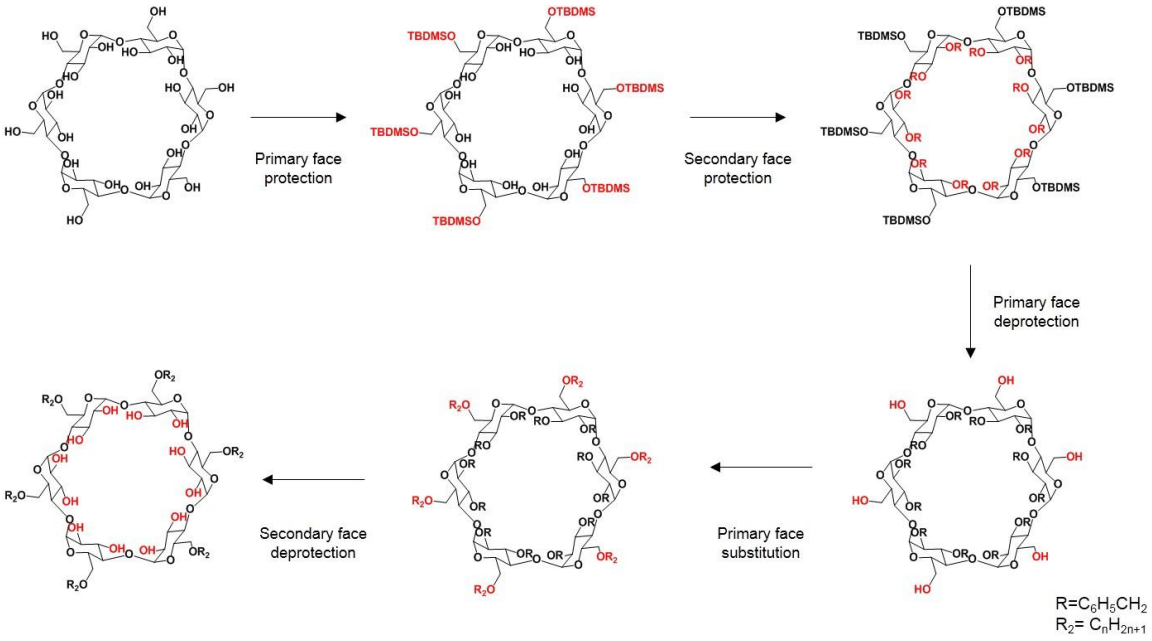


Figure 7 *Scheme of regioselective and random substitution of cyclodextrins. (n=6, 7 or 8)*

1. Primary face modifications

These reactions with strong electrophiles (such as alkyl, phosphoryl, sulfonyl or carboxylic acid chlorides) generally run under basic conditions, as CDs are stable in basic solvents but they decompose in the presence of strong acids. [75, 76] TBDMSCl is a popular reagent for performing primary face modification. In some cases, the 'long' reaction way is used for primary modifications, that consists of the following steps: protection of the primary, than the secondary face, deprotection of the C-6 positioned hydroxyls, and their substitution with the required group. [77] The last step is the deprotection of the secondary hydroxyl groups. (Scheme 1)



Scheme 1 *Selective substitution of the primary hydroxyl groups.*

Mono-substitution at the C-6 position

Mono-sulfonyl CDs are achieved by reacting 1 equivalent of benzene or *p*-toluenesulfonyl chloride with CD in pyridine or DMF containing base. [69] However, the use of pyridine is unadvised regarding its toxicity and ability to form complexes with CDs. It complicates the workup process, though it has the ability to orientate the reaction to the C-6 positon. [69] Mono-tosylation is a non-selective procedure, thus extensive purification is needed to gain the required product. [74, 78] Mono-6-tosylates are important intermediates of several derivatives, as a nucleophile can attack the electrophile carbon at the 6-position, thus the tosyl group can be replaced by suitable nucleophiles resulting e.g. iodo-, azido-, thio-, hydroxylamine- or alkylamino CDs. [69, 79-84]

Di-substitution at the C-6 position

Di-substituted derivatives are obtained by using more than one equivalent of reagent with CD. Due to the presence of possible positional and regioisomers, this process has enormous complexity. Neither the usage of an oversized reagent (e.g. mesitylenesulfonyl chloride) is effective in reducing this variety. [72, 85] Tosylation gives di-O-6, [86] di-O-2 [87] and di-O-3 [88] derivatives simultaneously, and the required product needs to be separated by reverse-phase column chromatography. The regiospecificity can be controlled by the utilization of the geometry of definite reactants. [89, 90] Similar to the mono-substituted ones, the di-sulfonated CDs are important intermediates of several di-substituted derivatives. [91, 92]

Tri-substitution at the C-6 position

Acyl or sulfonyl tri-substituted derivatives are prepared by reacting the respective acid chlorides in basic conditions. [93, 94] These highly electrophilic reactants attack indiscriminately either the primary and secondary hydroxyls, thus they result a mixture of products with diverse DS. The required compound can be obtained only after separation and purification with column chromatography. Tri-alkylated derivatives are synthetized by reacting alkyl halides with CDs in aqueous or DMF media, resulting again in a mixture of products. [69] The tri-alkylated derivatives are mostly used for chromatographic purposes. [95] In some cases (e.g. 6-triacetylated and trimethylated CDs), the *de novo* way of production from linear starting material is more favoured. [96, 97]

Per-substitution at the C-6 position

The selective modification of all primary hydroxyls is relatively easier, as per-substitution can be achieved when the reaction runs for longer time with appropriate amounts of reagents. [69, 98] Per-sulfonates are usually prepared from native CD with the addition of large amount of sulfonyl chloride in pyridine. Per-6-tosylated CDs are usually synthetized by using the ‘long’ method. [69, 99] Further per-substituted derivatives can be formed by displacing the sulfonate groups with nucleophiles (azides, halides, thiolate ions). Per-tosylated CDs with sodium halides in DMF give per-halogenated products. [99]

2. Secondary face modifications

The modification of the secondary hydroxyl groups is more challenging, as the secondary face is more crowded due to the presence of 12, 14 or 16 hydroxyl groups (α-, β-, γ-CD, respectively). Moreover, the intermolecular hydrogen-bond belt hampers further their

accessibility. Despite of these challenges, the already synthetized derivatives have important role in catalysis, enzyme mimics, etc. [69]

Mono-substitution at the C-2 position

With group transfer strategy, the production of mono-2-tosyl-β-CD was achieved. [70] Within group transferring, the tosylate is complexed in the CD cavity and the tosyl group gets transferred to the 2-position due to its orientation. [70] An advantage of the C-2 hydroxyls over the C-3 positioned ones, is that they are more acidic. It can be exploited when NaH is used as a strong base for selective tosylation. [67] In some cases, the best option is to choose the 'long' way synthesis and protect the C-6 hydroxyls before the tosylation at the 2-position. [69, 100] Esterification on this position is particularly challenging, however the production of ferrocene and pyrrolidine oxide containing mono-2-esters was reported using group transfer strategy. [101, 102] Reactions with alkyl halides are always indiscriminate, thus mono-2-alkyl derivatives are obtained by several challenging separation and purification steps, following the reaction of CDs with dialkyl sulfates in aqueous alkali solution. [103]

Di-substitution at the C-2 position

Regioisomers of di-2-substituted derivatives were achieved by the reaction of p-toluensulfonyl with CD in the presence of dibutyltin oxide. [104] 3-nitrobenzenesulfonyl chloride reacts with α-CD giving isomers of di-sulfonates at C-2 position, due to the formation of inclusion complex with the reagent. [87] α-naphthalenesulfonyl chlorides give exclusively 2-sulfonates with negligible amount of 3-substituted derivatives. [69] Selective dialkylation is very difficult [105], but dibenzyl derivatives have already been synthetized with 'long' way reaction. [106]

Per-substitution at the C-2 position

Per-substitution at the C-2 position is rather challenging, however by using strong base which selectively forms 2-alkoxide ions, the reaction with alkyl or sulfonyl chloride becomes possible. [68] When the DS increases, this selectivity loses significance. With the protection of the primary face, this problem can be excluded. [69] Per-2-tosylation is achieved by protecting the primary face with silyl group and then reacting the CD with large excess of tosyl chloride in pyridine on high temperature. [107] Per-alkylation can be possible also after protecting the primary hydroxyls, and using alkyl halogenides under basic conditions. [108] Although per-2-methylation is performed with methyl-iodide under anhydrous conditions in DMF with the presence of NaH. [68] The benefit of silyl group migration is exploited when

per-(2,6-di-O-TBDMS)-CD reacts with alkyl or benzyl chloride in strong base. After desililation 2-alkylated CD can be achieved. [109]

Mono-substitution at the C-3 position

Hydroxyls at the C-2 and C-6 positions possess higher reactivity than those at the C-3, thus their individual substitution is rather difficult. Most of the successful modifications (e.g. with thiolate ion) are obtained by using manno-mono-2,3-epoxycyclodextrin as starting material. [110-113] Tosyl chloride gives a mixture of 2-, 3- and 6-tosylates by reacting with native CDs, thus mono-3-tosylate needs to be separated by chromatographic methods. [114-116] The 2-naphthalensulfonyl and 3-nitrobenzenesulfonyle chloride shows higher selectivity to the C-3 position, due to their complex formation with CDs. [117]

Di-substitution at the C-3 position

Reacting CDs with sulfonating reagents can possibly give the di-substituted product, however in a mixture with other by-products. [87] Naphthalensulfonyl chloride forms complex with the CD, thus the sulfonyl group is directed to the C-3 position. [87, 88] They can be produced by the ring opening reaction of manno-di-2,3-epoxide with nucleophiles as well. [111]

Per-substitution at the C-3 position

Per-substitution is extraordinarily difficult. When the primary hydroxyls are protected, sulfonation will occur at the 2-position. [105] Sulfonyl chlorides fail to react with C-3 hydroxyl groups even if both the 6- and 2-positions are protected, as a result of steric hindrance and silyl group migration. [109] Although, per-2,3-epoxy-CDs are the intermediates in cycloaltrin formation. These cycloaltrins can be synthetized with a desired functional group at the 3-positions, when reacted with nucleophiles. [69]

Per-substitution at C-2 and C-6 position

As above mentioned, the hydroxyl groups at the C-6 and C-2 position have higher reactivity, thus their modification without affecting the C-3 position is achievable. Dialkyl sulfates react with CDs in DMSO-DMF in the presence of $BaO/Ba(OH)_2$. [118] Up to 12 carbon atoms per alkyl chain, the per-2,6-alkylation is feasible. [119, 120] Silylation is also possible in pyridine, when the reaction mixture is kept at high temperature. [121, 122] Interestingly, the presence of tosyl groups at the primary face decrease the reactivity of C-2 hydroxyls, thus per-2,6-tosylation is difficult [69], but per-acylation has been also performed. [123]

3. Complete per-substitution

Organic acid chlorides attack the three positions indiscriminately. Acetylation [23] and benzoylation [124] were achieved by using acetic anhydride and benzoyl chloride, respectively, and letting the reaction run for long time. Corresponding alkyl halides produce CD alkyl ethers. [34, 118]

4. Random substitution

When there is no pursuit of selective modification, random substitution is performed. Random modification results a mixture of molecules with modified hydroxyls on several positions. However, as it was previously described, regioselective procedures often produce a mixture of compounds, too. The difference is that while in this latter case separation has to be performed to distinguish and characterize the required product, in random modification the product is handled as a structural mixture. In practice, mostly random substituted CD derivatives are used.

The application of well characterized molecules does not necessarily offer such benefits which are worth the high-price, time consuming and sometimes inextricable accomplishment of separation procedures. For instance, the most common CD derivative ((2-hydroxypropyl)-β-CD) is a randomly substituted derivative, yet it is used even as an orphan drug or pharmaceutical excipient. [125]

Interestingly, the reactivity order of hydroxyl groups, depending on the used reactants, can differ. For example, during alkylation the secondary hydroxyls are more accessible then the primary ones, whilst in acylation the order follows the ‘original’ sequence (primary>secondary). [60]

Random modification has several advantages over regioselective derivative formation. The synthesis itself is more easy to perform (they are one step reactions or if more, they are simple). The reactions are easy to control, the time, temperature or equivalents can be changed target dependently. The yield in general is good, and the overall costs are lower (regarding the omission of expensive reactants and purification expenses). An important drawback is that the batch to batch reproducibility is difficult to achieve, it requires trusted and experienced crew. Furthermore, the final product’s DS can differ from laboratory to laboratory, despite of the exact same protocol. Added to this, the registration process for further pharmaceutical use is more complicated. [60]

Regarding selective modification, the analysis is usually easier to perform as the product is separated. Even though this approach happens to consist of several reaction steps, most of the common used intermediates are commercially available. Modifications can be performed during the process, and the procedures are reproducible. On the contrary, such methods result often lower yield, and they are more expensive. [60]

E. Polymers

Those derivatives which consist of two or more covalently linked CD units, are called dimers or polymers, respectively. Polymerisation is accomplished by one of the following methods: cross-linkage of CD rings, polymerizing bi- or polyfunctional substituent containing CDs or bonding CDs to other polymers. [126] In the first case, CD units bond to each other via the chains of the coupling agent, thus a three dimensional network forms. In the second case the CD residues are covalently bonded. [126] Up to a certain weight, polymers are water soluble, but as it increases, swelling or insoluble structures form. [45] (Figure 8) The areas most concerned in polymer utilization are chromatography and environmental industry. Currently, the polymer based hydrogels are in the focus of researchers' attention, as their ability of inclusion complex formation adumbrates several pharmaceutical potential. [127]

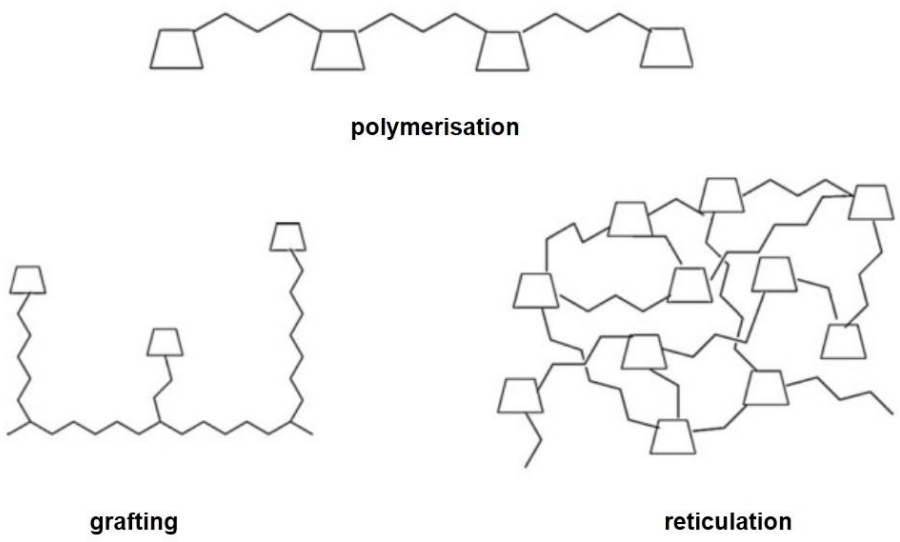


Figure 8 *Different types of insoluble, cyclodextrin containing polymers. Figure is adapted with modifications from [126].*

F. Purification and separation methods

The challenging separation and purification processes concerning CD chemistry can be explained by several factors. As previously described, in most reactions the crude compound contains a wide range of CD derivatives, which differ only in the number of substituents. Molecules exhibit e.g. 5 or 6 substituents often possess only slightly different physicochemical specificities, thus their separation requires careful consideration and exhaustive procedures. On occasion, these derivatives are impossible to separate with regular methods (column chromatography, recrystallization, precipitation). In these cases, HPLC can provide a solution.

Column chromatography

Column chromatography is a widely used technique in preparative chemistry. When a mixture of several components cannot be separated by usual methods like extraction or recrystallization, chromatography gives a solution. The main principal is the distribution of different substances between the stationary phase and eluent, thus the differential adsorption of components by the adsorbent. The adsorbent is usually silica or another highly polar material, containing several free hydroxyl groups. The eluent can be chosen from a wide range of organic solvents and their mixtures in carefully defined rate, to provide a less polar mobile phase. The utilization of pressure to force through the eluent on the stationary phase facilitates faster separation and it is called flash chromatography.

Highly hydrophilic or amphiphilic compounds cannot be separated and purified on normal phase, due to their retention on the also polar stationary phase. In this case, reverse phase column chromatography is performed, where the adsorbent is hydrophobic, usually long carbon chain bonded silica (e.g. C18, C8), and the mobile phase is a composition of polar solvents such as water, acetonitrile or methanol.

The access to CombiFlash® purification system provides possibility to perform separation and sample collection automatically. The theoretical background of this system equals with the manually performed column chromatography, however it allows to attain more rapid separation with better solvent consumption. [128]

Dialysis

It is a separation process in solution, driven by the different diffusion rate of molecules through a semipermeable membrane. Dialysis is used for a variety of purposes, such as small molecule removal from / introduction to a sample solution, but the most frequent application targets the elimination of unwanted small molecules, such as salts and other impurities. [129] When the

pore size of the membrane is carefully chosen, large molecules are retained in the sample, hence their diffusion is physically hampered. Small molecules though transfer through the membrane into the dialysate, driven by the pursuit to reach equilibrium between the two chambers. (Figure 9) After reaching the equilibrium and the dialysate is changed, the procedure starts again, thus the quantity of pollutant can be decreased to an acceptable or negligible level. Dialysis membranes are usually made from cellulose or cellulose esters, and their main characteristic is the molecular weight cut off. This number defines the smallest molecular mass which is restrained, thus cannot diffuse effectively through the membrane. As diffusion is not a one direction process, molecules can transfer from the dialysate to the sample too, thus the composition of the dialysate shall be carefully chosen. When pure solvent is used only one-way diffusion will occur. Dialysis is a slow procedure depending on the diffusion rate of different molecules, but it can be accelerated by continuous stirring and using at least 10 times volume of dialysate.

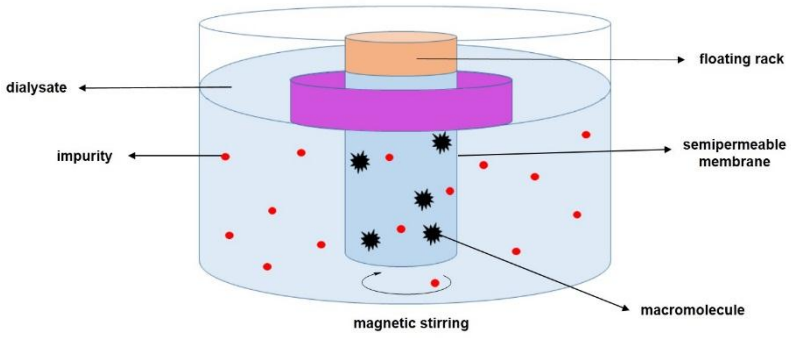


Figure 9 *Procedure of dialysis.*

Celite

Celite® or diatomaceous earth is an often used filtration aid and adsorbent to remove particulate impurities, salts and clarify solutions. It retains fine particles which would easily pass through a paper or glass filter. [130]

Precipitation

Substances can be individually separated from solution by converting them into an insoluble form. Following the addition of a precipitant, the by-products, impurities and other undesired compounds will stay in the solution with the exception of the clear compound, which can be separated by filtration. [131]

III. Cyclodextrin applications

A. Food industry

Their natural origin provided a particular position to CDs from the early times. In Japanese tradition, natural products have high respect and preference for industrial use, and CDs were declared by the Japanese scientific community as 'non-toxic' compounds, and soon Japan became the world's first CD producer and consumer. [17, 51] They were also the first who authorized the application of α- and β-CDs as food additives in 1976. In Hungary in 1983 β-CD was approved for stabilization of natural flavours, then in France in 1986 also β-CD gained limited authorization as a flavour carrier. By now, the parent CDs are registered in the Codex Alimentarius of Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2013) with the INS number of 457, 459 and 458 (α-, β-, γ-CD, respectively). [132] β-CD is registered in the EU as E-459 additive. [133]

For decades, the general consideration was that CDs do not occur in the nature. [134] However, in 1986, three O-acetyl-O-butyryl-O-carbamoyl-O,O-dimethyl-α-CD derivatives were found in *Tolypothrix byssoidea*, an epilithic, aerial blue-green alga. [135] The found CDs with unusual structure demanded the modification of the general point of view. The original expectation was to find and eliminate *native* CD forms, however as Entzeroth pointed out, that naturally occurring CDs probably present in a more complex structure, due to the enzymatic production. Enzymatically modified CDs are called branched CDs, they contain maltosyl or glucosyl groups, and their production is also possible by thermal treatment. [136] Later Szente *et al.* reported the presence of branched CDs in enzyme- and heat-processed starch containing foods. [134]

CDs are suitable food supplements due to their versatile benefits. They are tasteless, odourless, non- or partly digestible, non-caloric saccharides. They act like prebiotics in the human gastrointestinal tract, as they can be fermented by the natural microflora. In the presence of CDs, the absorption of lipids and carbohydrates are limited. They reduce the glycaemic index, hence CD application can be a promising approach to lower the risk of diseases resulted by high blood glucose level (diabetes, hyper-lipemia, hypertension and atherosclerosis), in sports nutrition or in the management of glycogen storage disorders. Common functions that CDs fulfil as food additives are the following:

- When the aim is to reduce the cholesterol content, native β-CD is applied in prior, because β-CD-cholesterol complexes are insoluble, thus the precipitate can be easily eliminated.

[6] Diary production took advantage on this phenomenon, aiming cholesterol removal from eggs [137], milk, butter [138], whipping cream [139] or several chees types [140, 141]. When hens were fed with β-CDs, they laid reduced cholesterol eggs. [133]

- Phytosterols (e.g. β-sitosterol, camposterol, stigmasterol) compete with cholesterol absorption from the intestines. When complexed with CD, their solubility, thus their bioavailability increases. It is a favourable way to lower the cholesterol intake. [142, 143]
- Poly-unsaturated fatty acids are important nutraceuticals, and they are sensitive to autoxidation. [144] The oxidized substances can be harmful, or provide undesirable smell and taste. The complexation of PUFAs within CDs omits the addition of further antioxidants, and enhances the bioavailability. [133]
- Elimination of undesired factors (e.g. unpleasant smell of goat milk products, presence of allergens, mycotoxins and aflatoxins, hindrance of bitter taste, etc.). [145-150]

Orally administered α-CD absorbs in very low rate (less than 1%), and it occurs mostly after their metabolism in the colon. Due to this, α-CDs are respected as dietary fibres, and prebiotics. [133] To decrease the blood sugar rise after carbohydrate containing meal, 5g of α-CD is recommended to take, per 50g of starch. [151] α-CD also hampers the lipid absorption, as they prevent their degradation, thus they may improve the blood's fatty acid profile. [152] Based on these advantages, α-CD is used as a dietary fibre in the U.S and Canada under the name of FBCx and in Australia as Calorees. [153] It is said to be the first soluble dietary fibre, which helps to manage weight loss and ameliorate blood lipid levels (total and LDL cholesterol, Apo B) without changing one's normal diet. [154, 155]

Food packaging

In general sense, food packaging operates as a passive barrier, which hampers the interaction between the food and the environment. [133] Active packaging maintains food preservation by balancing the interaction between the food product, the packaging material and the environment; thus serves the extension of shelf life and food safety. [156] CDs are suitable to incorporate into the packaging film. When the CD is empty, penetrating volatiles, pollutants or escaping aroma substances can be captured within their cavity. [157] The complexation of natural preservatives, antimicrobial or antioxidant components, and their humidity induced or sustained release facilitates the minimisation of preservative consumption. [158] Intelligent packaging gives visible information of weakened barrier function, e.g. by using CD-dye

complex, where the coloured compound gets released by a competitor (e.g. humidity) causing colour change. [159] CDs are also often used in tea of coffee bags to prevent aroma loss. [133]

B. Cosmetic industry

The world-wide trend, demanding the application of natural substances with no artificial preservatives added, is highly significant in the cosmetic industry. As the consumers are looking for innovative formulations produced by the latest technology, more and more technologies are used in the beauty industry which were originally applied in the pharmaceutical production. It was soon recognized, that the beneficial inclusion complex forming ability of CDs can be exploited also in cosmetic formulations, hence the number of CD-based products are increasing. An important advantage of CDs is that they are not nutrient medium for microorganisms, which minimizes the necessary amount of preservatives. The following examples show some of the various functions which can be fulfilled by CDs. [160, 161]

- **Protection of guest molecules:** the stability of light and heat sensitive active ingredients (e.g. tea tree oil, hydroquinone, kojic acid, tocopherols) can be increased by inclusion complex formation. The unfavourable interactions of incompatible ingredients are also often avoided by their complexation. [160, 162]
- **Solubilisation:** The complete or partial complexation of poorly soluble active substances increases the aqueous solubility without using organic co-solvents. (e.g. salicylic acid, menthol, triclosan, retinol). [160, 162]
- **Elimination of undesired compounds:** In some cases, it is necessary to mask the undesired smell of the active ingredient (dihydroxyacetone, Matricaria oil), which can be achieved by CD complexation. [160] On the other hand, CD-scent complexes are used in deodorants, where the fragment molecule is replaced by the odorous molecule. [162]
- **Improvement in handling:** Some liquid substances (α-tocopherol, vitamin-E, peroxyacetic acid) form solid complexes with CDs. In solid state their formulation is easier, as they can be used as powders or suspensions in creams and lotions. [160, 162]

The main disadvantage of CD utilisation in cosmetic formulas is that CDs are able to complex functional components, such as preservatives, which reduces the antimicrobial activity. To eliminate this problem, the choice of preservative shall be carefully chosen, and the re-calibration of functional preservative concentration should be done after CD application. [163]

C. Textile industry

CDs are widely used in the textile industry on several levels. [164]

1. Removing detergent traces and as defoaming agents. When CD is added meanwhile rinsing, it fixes the last traces of detergents which reduces the water consumption. When a highly volatile scent molecule is complexed within the CD cavity its sustained release provides pleasant odour for long term.
2. In textile colouring, CD can bind dye molecules. The affinity of the fibre to the dye is increased and the dye's diffusion coefficient to the fabric decreases. CDs also make possible homogenous colouring with poorly soluble dyes (e.g. disperse dyes). CD containing washing liquid prevents the running of colours. At the end of the dyeing procedure, surfactants can be specifically captured by them, thus the water can be recycled.
3. Chemical finishing provides functionalities to textiles and makes them appropriate for special applications, also improves their appearance. [17] When CD is attached to the textile fibres, it prevents the penetration of sweat into the textile fibre, also aids their easy removal. O-methoxycinnamaldehyde-β-CD complex incorporated in shoe insoles reduced the undesired symptoms of patients with athlete's foot. Treatment of hospital bedsheets with volatile antimicrobial-CD complex containing agents may be a part of infection prevention. When the macrocycles are complexed with insecticides (e.g. isobornyl thyocianoacetate) a long term insecticidal effect can be achieved. CD can play an important role also in wrinkle-controlling.

CD grafted fibres contain permanently fixed CD molecules (bonded by e.g. glyceryl ether, polycarboxylic acid or triazinyl). [164-166] The release of the active ingredients (volatile scent, BAM, etc.) penetrated the cellulose is immediate, while the complexed molecules' release is sustained. (Figure 10)

The complexation of organic substances excreted via skin may serve medical diagnostic purposes. Textiles with CDs can bind sweat substances, which facilitates their detection and identification. [167]

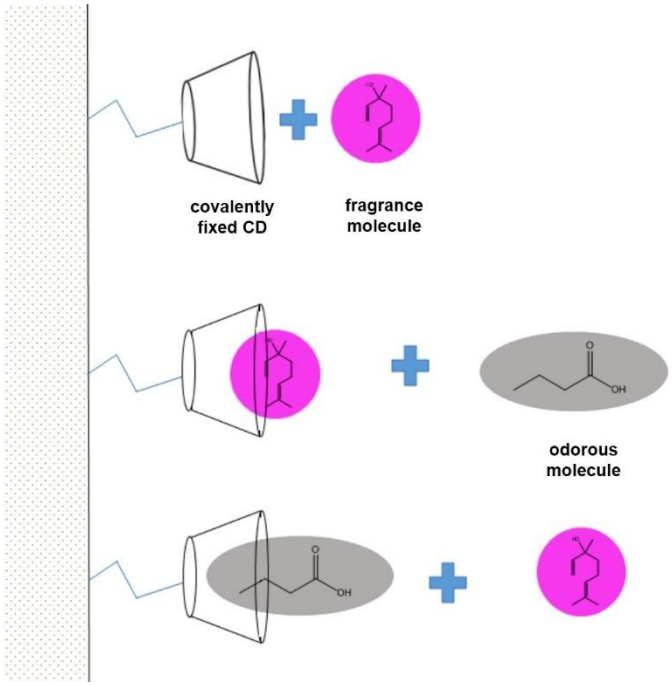


Figure 10 *Mechanism of guest molecule exchange in textile materials maintaining covalently bonded CD. When the malodour molecule has higher affinity for the CD cavity, it gets entrapped meanwhile the release of the scent molecule provides pleasant smell.*

D. Biotechnology

Bioconversion and fermentation processes are often limited by the poor solubility of organic substances. The production procedure is also frequently hampered by the inhibitory or toxic effect of substrates or products on the biocatalyst. CDs increase the solubility of complexed guest molecules, moreover they do not affect enzymes or damage microorganisms. Thus, in their presence enzymatic conversion of lipophilic substrates can be performed at higher dissolved substrate concentration. This environment helps to diminish the toxicological and inhibitory effects as well. [168] However, the fermentation environment is often harsh, CD utilisation is still rewarding as the macrocyclic ring is stable up to 200°C, and depolymerisation only occurs at extreme pH. CDs have been successfully applied in the following cases:

- The production of lankacidin antibiotics (macrolides like lankacidin A or C and lankacidinol A and C) by *Streptomyces rochei volubilis* was increased in the presence of either α-, β- and γ-CD. [169] The biosynthesis of podophyllotoxin (antiviral, anthelmintic, purgative, cathartic, vesicant) is also positively influenced in the present of β-CD. [170]
- The vaccine mixture used against diphtheria, pertussis and tetanus contains pertussis toxin. It is produced by *Bordatella pertussis*, but the fermentation procedure is challenged by the

high sensitivity of the bacteria. The addition of 0.5mg/ml DIMEB increased the production to 100 folds and had positive effect on cell growth as well. [170, 171]

- In several cases, the presence of β-CD or its derivatives increased the production yield of steroid transformation, such as hydrocortisone to prednisolone (by *Anthrobacter simplex*), 17-β-estradiol to 4-hydroxy-estradiol (by phenoloxidase enzyme), cholesterol or β-sitosterol to 4-androstene-3-17 and 1,4-androstadiene-3-17-dione (by *Mycobacterium sp*.), or cholesterol to testosterone (by *Lactobacillus bulgaricus*). [170, 172]

E. Pharmaceutical applications

After decades of intensive, wide-range research, CDs are now actively used pharmaceutical ingredients all over the world. The monographs for all the three native CDs can be found in the Japanese Pharmaceutical Codex and United States Pharmacopeia and National Formulary. α- and β-CD are listed in the European Pharmacopoeia under the name of Alphadex and Betadex, respectively There is a monograph for HPBCD in the Eur. Pharm. and USP/NF, while the monograph for SBEBCD is in the USP/NF. HPBCD, SBEBCD and γ-CD are registered by FDA as Inactive Pharmaceutical Ingredients. The FAO/WHO Committee of Food Additives recommends an Acceptable Daily Intake of 5 mg/kg/day for β-CD. Due to the more favourable toxicological profile of α- and γ-CD, no ADI was defined for them. The not specified ADI value is the best specification, as it is limited to compounds with really low toxicity. [173, 174]

CD as complex forming excipient

CDs have gained importance in the following areas:

- solubility, bioavailability and stability enhancement,
- reduction of undesired odours and tastes,
- retention of volatile substances,
- ameliorating the toxic effect of the active ingredient,
- enabling liquids for solid state formulations,
- preventing incompatibilities of concurrently present moieties, etc.

Numerous reviews, books and articles are dealing with the pharmaceutical applications of CDs. (Figure 11) This thesis is not intended to introduce this enormous work in details, although the cited publications may be interesting for further survey. [51, 173, 175-180]

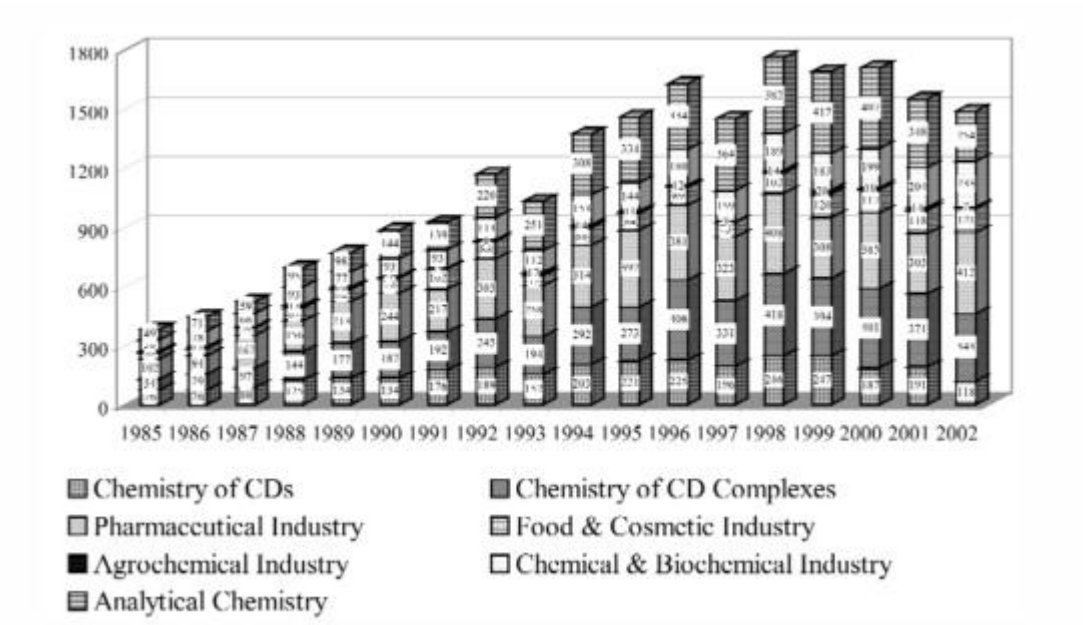
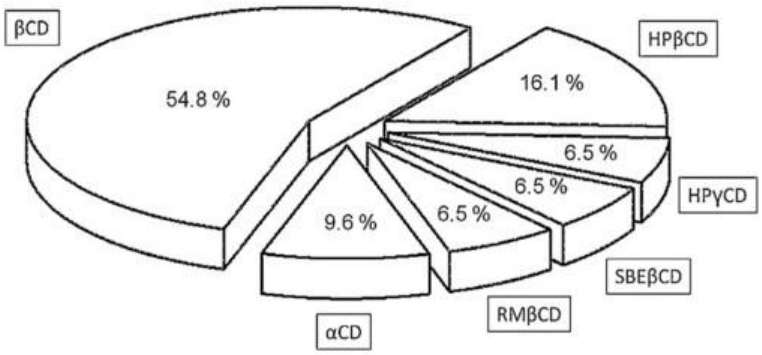


Figure 11 *The increasing number of CD-related publications between 1985 and 2002. This tendency has not stopped since then, but further escalades. Figure is adapted from [11].*

Recently, approximately 35 CD-containing products are available on the market. (Table 2) The top three most frequently used CD derivatives in these formulations are the native β-CD, HPBCD and α-CD, respectively. (Table 2) The reason of the frequent application of β-CD is the ease of its production and the affordable price. However, several of its derivatives possess improved physicochemical properties. For instance, HPBCD has more favourable dissolution and toxicological profile, resulting access to parenteral administration. [174]

Table 2 *Distribution of different CD derivatives applied in commercially available pharmaceutical formulations, and marketed CD-based pharmaceutics. Figure and table are modified from [174, 178].*



Drug/cyclodextrin	Trade name	Formulation	Company (country)
α-Cyclodextrin (αCD)			
Alprostadil	Caverject Dual	Intravenous solution	Pfizer (Europe)
Cefotiam-hexetil HCl	Pansporin T	Tablet	Takeda (Japan)
Limaprost	Opalmon	Tablet	Ono (Japan)
PGE1	Prostavastin	Parenteral solution	Ono (Japan); Schwarz (Europe)
β-Cyclodextrin (βCD)			
Benexate HCl	Ulgut, Lonmiel	Capsule	Teikoku (Japan); Shionogi (Japa
Cephalosporin	Meiact	Tablet	Meiji Seika (Japan)
Cetirzine	Cetrizin	Chewable tablet	Losan Pharma (Germany)
Chlordiazepoxide	Transillium	Tablet	Gador (Argentina)
Dexamethasone	Glymesason	Ointment, tablet	Fujinaga (Japan)
Dextromethorphan	Rynathisol	Synthelabo (Europe)	
Diphenhydramine and chlortheophylline	Stada-Travel	Chewable tablet	Stada (Europe)
Ethinylestradiol and drospirenone	Yaz	Tablet	Bayer (Europe, USA)
Iodine	Mena-Gargle	Solution	Kyushin (Japan)
Meloxicam	Mobitil	Tablet and suppository	Medical Union (Egypt)
Nicotine	Nicorette	Sublingual tablet	Pfizer (Europe)
Nimesulide	Nimedex	Tablets	Novartis (Europe)
Nitroglycerin	Nitropen	Sublingual tablet	Nihon Kayaku (Japan)
Omeprazole	Omebeta	Tablet	Betafarm (Europe)
PGE2	Prostarmon E	Sublingual tablet	Ono (Japan)
Piroxicam	Brexin, Flogene, Cicladon	Tablet, suppository	Chiesi (Europe); Aché (Brazil)
Tiaprofenic acid	Surgamyl	Tablet	Roussel-Maestrelli (Europe)
2-Hydroxypropyl-β-cyclodextrin (HPβCD)			
Cisapride	Propulsid	Suppository	Janssen (Europe)
Indometacin	Indocid	Eye drop solution	Chauvin (Europe)
Itraconazole	Sporanox	Oral and intravenous solution	Janssen (Europe, USA)
Mitomycin	MitoExtra, Mitozytrex	Intravenous infusion	Novartis (Europe)
Sulfobutylether β-cyclodextrin sodium salt (SBEβCD)			
Aripiprazole	Abilify	Intramuscular solution	Bristol-Myers Squibb (USA); Otsuka Pharm. (USA)
Maropitant	Cerenia	Parenteral solution	Pfizer Animal Health (USA)
Voriconazole	Vfend	Intravenous solution	Pfizer (USA, Europe, Japan)
Ziprasidone mesylate	Geodon, Zeldox	Intramuscular solution	Pfizer (USA, Europe)
Randomly methylated β-cyclodextrin (RMβCD)			
17β-Estradiol	Aerodiol	Nasal spray	Servier (Europe)
Chloramphenicol	Clorocil	Eye drop solution	Oftalder (Europe)
γ-Cyclodextrin (γCD)			
Tc-99 Teboroxime[a]	CardioTec	Intravenous solution	Squibb Diagnostics (USA)
2-Hydroxypropyl-γ-cyclodextrin (HPγCD)			
Diclofenac sodium salt	Voltaren Ophtha	Eye drop solution	Novartis (Europe)
Tc-99 Teboroxime[a]	CardioTec	Intravenous solution	Bracco (USA)

As Szejtli stated: *inclusion complex formation proceeds by an energetically favoured interaction of a relatively non-polar guest molecule with an imperfectly solvated hydrophobic* (in comparison with water) *cavity*. [45] This 'energetically favoured interaction' is driven by secondary chemical interactions, such as van der Waals electrostatic forces or hydrogen bonds.

The guest molecule ought to be suitable in size with the cavity dimensions, but adequate conformity in polarity also plays a main role in complex formation. Although, significantly larger molecules often happen to form complex with a CDs, when only a suitable molecular group or side chain penetrates into the cavity. [45] The guest molecule accommodates in the cavity endeavouring the maximal contact between the relatively hydrophobic parts of both the CD and guest.

The inclusion complex formation is a reversible procedure. The molar ratio in solution is usually 1:1, however other host-guest complexes, such as 1:2, 2:1, 2:2 can co-exist. When the guest molecule is too large to accommodate within one cavity, and its other end is also preferable for encapsulation, it can be entrapped in a second host, thus establish a 2:1 CD-drug complex. With regard to the position of the host molecules, contact can be determined between the secondary, the primary, both phases. On the other hand, when the cavity size exceeds the guest molecule's dimensions, 1:2 host-guest ratio inclusions might be formed. [46] It should be noted, that the CD complexation effects a drug's pharmacokinetics only, when the binding constant is greater than $10^5 M^{-1}$, otherwise it does not hamper the therapeutic effect of the guest molecule. [181]

The complexation efficiency (CE) is in strong correlation with the intrinsic solubility (S_0) of a drug and the stability constant ($K_{1:1}$) of a drug-CD complex. [182] CDs can be used as solubility enhancers only with those drugs, which have relatively high CE. [178, 183] Enhancement in S_0 often results the decrease of $K_{1:1}$, and the contrary might also occur. Nevertheless, until the increase exceeds the value of decrease, the result in all cases is CE enhancement. Ionization (phenytoin, naproxen, naringenin, alvocidib) and salt formation (carvedilol, manidipine, aconazole, naproxen) are the most often applied techniques with the same purpose: increasing the S_0. [183] Water soluble polymers (such as HPMC, PEG, NaCMC, HDMBr), are well known for enhancing the solubility of CDs and CD-complexes (with acetazolamide, carbamazepine, celecoxib, dexamethasone, famotidine, irbesartan, etc.). [183] In low concentration, they result CE enhancement due to an increase in $K_{1:1}$. Addition of an organic cosolvent (e.g. ethanol, methanol, n-propanol) can increase S_0 by reducing the hydrogen-bond density in the aqueous media, but in the same time it decreases $K_{1:1}$ due to the reduced polarity

of the aqueous environment. [184] Charge-charge attractions may be exploited when an oppositely charged CD and drug molecule are complexed. The poorly soluble ziprasiodone and its various salts were compared in complex with HPBCD (uncharged) and SBEBCD (negatively charged). The charge-charge interaction in case of SBEBCD significantly increased both $K_{1:1}$ and CE. [183, 185]

Ternary CD complexes are those of supramolecular systems, which contain a third component beside the API and CD. The origin of the third component can differ with respect to its purpose, which can manifest in enhanced physicochemical, transport properties or bioavailability, or the reduction of the necessary amount of CD. [174] Often used ternary components are: metal [186] and organic ions [187, 188], polymers [174], a second API with similar pharmaceutical effect [189], or a second type of CD [190, 191], moreover, the application of phosphatidylcholine also provided promising results [192].

Metabolism

CDs are less accessible for enzymatic hydrolysis than linear dextrins. It is due to the cyclic structure and that the oxygen bonds are buried within the cavity. α-amylase hydrolyses starch from within the chain, and it can attack CDs, too. The enzymatic degradation is slower when the CD is in complex, and it also depends on the dimension of the macrocyclic ring. γ-CD is rapidly digested by pancreatic and saliva α-amylases, while α- and β-CD are more resistant, and they are more likely degraded by the bacteria of the lower intestinal tract. α-CD is more resistant than β-CD. Parenterally administered CDs are excreted mainly via glomerular filtration (more than 90%), the rest is eliminated by other pathways such as liver metabolism, biliary excretion, etc. [174, 193]

The pharmacokinetics of the three CDs are very similar, also to those non-cyclic dextrins with similar molecular weight. The $t_{1/2}$ of the elimination range is between 1.4 and 2h. After parenteral administration, 90% of the CDs are eliminated in 6h, and 99.9% within 24h, thus no accumulation is observed (except when a patient suffers from renal failure). The volume distribution (V_D) is approximately 0.2L/kg. [193, 194]

Cyclodextrin as active ingredient

In 2013, orphan designation was granted to HPBCD by both the European Commission and FDA, for the treatment of Niemann-Pick disease, type C. [195] The Niemann-Pick disease is an inherited autosomal recessive lysosomal storage disorder, which causes the accumulation of un-esterified cholesterol. It manifests in sever progressive neurodegeneration (behavioural problems, learning disabilities, difficulty in moving and speaking) and the enlargement of the liver and spleen. [196] HPBCD is proved to be a promising treatment due to its ability to sequestrate excess cholesterol from the organs. After intravenous HPBCD administration, the treatment seems to be effective in improving hepatosplenomegaly and central nervous system deficits.

A γ-CD derivative, namely Sugammadex (6-O-3-carboxypropylthio)-β-CD was approved first by the European Union in 2008 (also in Norway and New Zealand) and by the FDA in 2015 as a neuromuscular reversal drug. [197] During the intraoperative period, muscle relaxants are used to facilitate the endotracheal intubation and to ensure the patient immobility. To terminate the neuromuscular blockade, reversal agents are used, which have to fulfil several requirements. The ideal blockade reversal agent works with a fast onset, should be effective any time, regardless to the relapsed time since the relaxant administration, also should have high affinity to the relaxant, and ought to provide complete reversal. Sugammadex (under the trade name of Bridion®) as a complex forming agent effectively binds rocuronium, vecuronium and pancuronium at 1:1 ratio, however it has the highest affinity to rocuronium. The binding constant of Sugammadex-rocuronium complex is $10^7 M^{-1}$. [198] Sugammadex is administered intravenously, and two types of dosage forms are available: 200mg/2ml and 500mg/5ml.

IV. Toxicology

A lot of molecules are synthetized in chemistry laboratories or designed *in silico* for their expected human or veterinary use. Preclinical studies mediately provide assurance of their safety and efficacy. Safety evaluation is a primary concern not only for drug candidates but for excipients too, as several substances have the propensity to trigger adverse effects. Pharmaceutical excipients are handled as inert substances, although their simultaneous presence can often result in drug-excipient or excipient-excipient interactions, which might be advantageous (e.g. solubility enhancement) or even harmful (e.g. allergic reaction). Thus, the profound safety screening of excipients such as CDs, is being indispensable, including their pharmacokinetic and toxicological profile.

A. *In vitro* evaluation

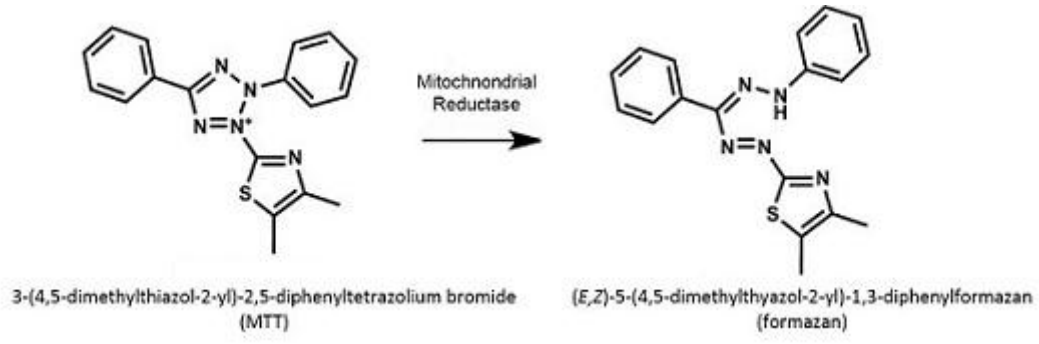
The preclinical phase of drug development consists of *in silico*, *in vitro* and *in vivo* stages. The first two sub-processes are intended to exploit the efficacy and toxicological profile of certain substances, to allow only those candidates for further safety evaluations, which seem to have no harmful effect on this level. Due to these precursory studies, the sum of *in vivo* tests is minimized. [199]. The existing and increasing social concern about animal tests, especially which may cause pain and suffering, urges the replacement or decrease the usage of animal models. *In vitro* assays can possibly include subcellular systems (macromolecules, cell organelles, subcellular fractions), cellular systems (primary, immortal cell lines, genetically modified cells, cells in different stages of differentiation, stem cells, barrier systems or co-cultures), whole tissues and perfused organs. [200] Non-animal (computer based or *in vitro)* tests help to enhance the extrapolation from *in vitro* to *in vivo* in human, as animal tests have questionable relevance to human conditions. The careful evaluation so called *interspecies extrapolation,* of *in vitro* tests performed on animal and human derived cells and tissues, then the extrapolation of these consequences to *in vivo* level, provides basis for assessing the risk factors and possible outcomes in case of human exposure (parallelogram approach by Blaauboer *et al*., 1990). [201]

Cytotoxicity is considered as the potential of a certain compound to induce cell death, as a consequence of damaging basic cellular functions. Further investigations can thoroughly define the type of toxicity based on its dose and time dependency, the effect on the cell cycle, or the reversibility. [200] Due to the *in vitro* cytotoxic evaluations, the concentration range of safety application can be determined. Each compound can be defined by a concentration value which

affects 50% of the certain cell population. For instance, IC_{50} refers to the half maximal inhibitory concentration, HC_{50} to the concentration which causes 50% hemolysis, or TC_{50} to the concentration which toxic for 50% of the examined population. By the comparison of these values, different substances can be ranked, thus the safest compounds may be easily chosen. Furthermore, the quantification of the effect of a single component in different systems is also possible.

MTT assay

MTT assay is a broadly used, rapid colorimetric method to measure the *in vitro* cytotoxicity of certain compounds on cell lines or primary cells. It is usually performed in 96-well plates, thus a high throughput method, which excludes cell counting. MTT assay is based on the transformation of MTT dye to a formazan crystal. By assessing cell metabolic activity, it reflects the viable cell number. NADPH-dependent oxidoreductase enzymes are able to convert MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into its formazan form ((*E,Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan). (Scheme 2) MTT is cleaved only by metabolically active cells, but not by dead cells, neither erythrocytes. The amount of transformed formazan is thought to be directly proportional to the number of living cells. [202] For decades it was considered as an evident, that MTT is transformed in the mitochondria, however in the last few years, assumptions arose which doubt the mitochondrial localization of the formazan formation. [203, 204]



Scheme 2 *Conversion of MTT tetrazole to a formazan salt by mitochondrial reductase enzyme.*

The transformation is indicated by colour and solubility changes, as the yellow tetrazole is water soluble, but the formazan salt is purple coloured and not soluble in the culturing media or buffer solution. Due to the colour change, this method is also applicable for quick qualitative evaluation. When the formazan salt is solubilized in the appropriate mixture of acidified

alcohol solution, the absorbance of the coloured solution can be quantified at 570nm. [205] When the effect of a certain compound in different concentrations is registered correlated to the untreated control, the IC_{50} values can be easily calculated. [202]

Real-Time Cell Electronic Sensing (RT-CES)

RT-CES is a label-free, non-invasive technique for the real-time monitoring of cell growth, adhesion, necrosis and apoptosis. [206] The system uses specially designed plates containing interdigitated gold microelectrodes, which monitor non-invasively the viability of cultured cells. [207] The electrical impedance is measured dynamically, which readout value is driven from the interactions between the golden electrodes and adherent cells. EI correlates linearly with the cell index, which refers to the cell number, adherence and cell growth. [207] It can be calculated for each time point by applying the $(R_n-R_b)/15$ formula (R_n – EI of the well, when it contains cells, R_b – EI of the well, when it contains only media). [208] RT-CES is appropriate for the verification of end-point viability assays (e.g. MTT), also allows the determination of optimal time points to perform standard cell viability assays. [209] The continuous monitoring of cell viability provides extended data on the kinetics of cytotoxicity, thus not only verifies but complements the end-point assays. The tested compounds are used in different concentrations and the assays are performed for the whole length of experiment.

Figure 12 summarizes the benefits and drawbacks of both MTT cell viability assay and RT-CES method.

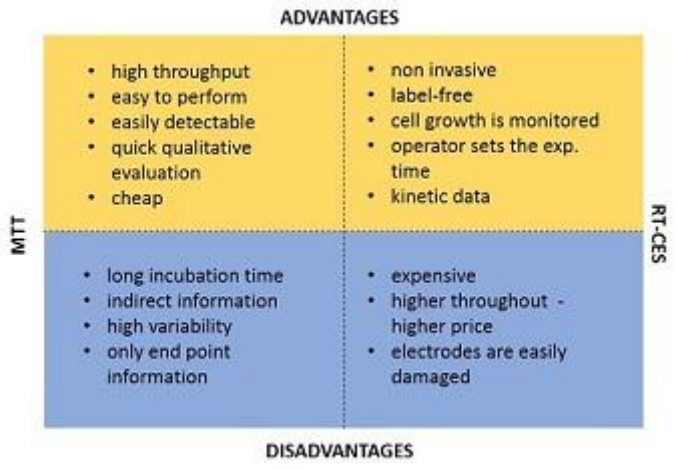


Figure 12 *Comparison of MTT assay and RT-CES.*

Hemolysis test

Drug related hemolysis is rare, it mostly occurs at high drug concentrations. Although, some compounds may induce hemolysis at low concentrations in patients who are genetically predisposed. [210] The FDA recommends the *in vitro* evaluation of pharmaceutical excipients intended to *intravenous* administration, for the exploration of their haemolytic potential. [211] *In vitro* hemolysis assay is usually performed on freshly taken, healthy animal or human blood erythrocytes. A defined amount of RBC is incubated with the given compound. The haemoglobin which diffused into the extracellular environment due to hemolysis, remains in the supernatant after centrifugation. The assay is measured at 540nm, and the absorbance value correlates with the free haemoglobin content, thus the extent of hemolysis. When a certain compound is tested in different concentrations, the HC_{50} value can be determined, correlated to complete hemolysis in water. This value provides a tool for ranging drugs, excipients or other substances by their haemolytic activity.

Caco-2 cell line

Both *in vitro* and *in vivo* methodologies have several advantages and drawbacks. Cell lines used for *in vitro* assays:

- have cancerous origin, thus they are transformed, or
- are driven from normal cells, thus they become immortalized, or
- are primary cells, so they can be kept in a culture only for limited time.

In each case, it has to be considered, that these cells are isolated from their natural environment. Their survival rate is limited, they are not integrated to a tissue or organ topology, the intercellular connections are severely reduced, and their metabolic competence is imbalanced. [200]

The human intestinal Caco-2 cell line has been obtained 72 years ago from a human colon adenocarcinoma of a Caucasian male patient. [212] Under culturing circumstances these cells spontaneously differentiate to form a polarized epithelial cell monolayer. Both the morphological and biochemical characteristics resemble to the enterocytes of the intestinal mucosa, which might be surprising, with regard to the colonic origin. Differentiated Caco-2 cells possess cylindrical polarized morphology, with microvilli on the apical side and tight junctions between the adjacent cells. [213] (Figure 13) Due to the tight intercellular connections, Caco-2 monolayer forms a physical and biochemical barrier to the passage of ions

and molecules. The characteristic semblances were also confirmed by the enzymatic activity of those hydrolase enzymes which are typically associated with the brush border apical microvilli of the small intestine (e.g. sucrase-isomaltase, alkaline phosphatase, aminopeptidase). [214] Caco-2 cells are mainly used as a monolayer rather than individual cells, however several assays are performed prior to reach complete integrity, such as end point or non-invasive cell viability assays (MTT assay, LDH test, RT-CES, etc.). These methods may assist in the exclusion of those false presumptions, which explains absorption enhancement with the disruption of intestinal mucosa.

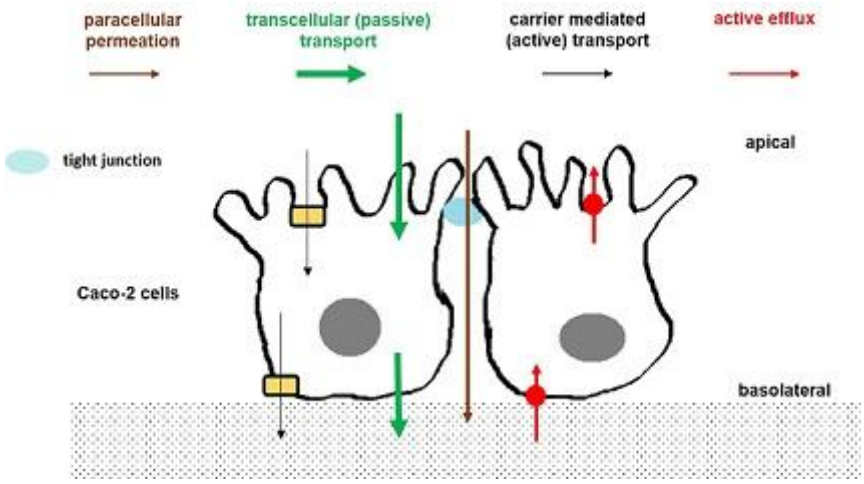


Figure 13 *Caco-2 monolayer with morphological and biochemical characteristics, similar to small intestinal mucosa.*

The biological complexity of *in vivo* absorption process challenges its *in vitro* interpretation. In the 1990's, the model based on Caco-2 cell monolayer have rapidly gained an exclusive popularity as it seemed an ideal adaptation method. [215, 216] Despite of the favourable differentiation patterns under culturing circumstances, the question of indeed trustable prediction has reasonably arisen. Complicating factors are the permanent activity of gastric fluids, or the presence of p-glycoprotein and other transporters, limiting the uptake of certain substances. As the Caco-2 cells have cancerous origin, they are well known to overexpress Pgps, thus the efflux activity is overactive, compared to the healthy conditions in human. [217] Yee *et al*. have carried out a comprehensive study to survey the predictability of absorption procedure across Caco-2 cell monolayer. [218] He concluded that Caco-2 cell based models have good correlation with *in vivo* human absorption, regarding all the three possible absorption mechanism. The selection procedure of candidates for further clinical studies shall include a Caco-2 based surrogate to reduce the attrition rate. To reach the highest correlation rate between the Caco-2 model and the small intestine, the culture age and the range of passages

have to be also considered. [219] It has been shown, that the rate of transporter protein expression varies with the age, but stable expression rate was observed within a broad range of passages (29-43). [219]

Recently used *in vitro* systems do not combine the modelling of the two crucial pitfalls of bioavailability, namely the absorption and hepatic clearance. [220] Hybrid systems might provide a solution for a better optimized screening. Lau *et al*. have developed a complex system consisted of a Caco-2 monolayer and cryopreserved hepatocytes in different compartments separated by a polyethylene membrane. [221] Those substances which had been absorbed via the cell monolayer, were exposed directly to the first-pass metabolism, thus these two determining factors were simultaneously predicted. This hybrid system showed satisfactory correlation with *in vivo* bioavailability results, however it still does not take into account those cases, when the drug is eliminated by extrahepatic metabolism.

Caco-2 transport model

FDA accepts *in vitro* permeation studies via epithelial cells, as a reliable method to determine permeability. [3] The transport model builds up from an apical and basal chamber, representing the intestinal lumen and submucosal environment, respectively. Caco-2 cells are grown on a semipermeable polycarbonate membrane, representing the intestinal endothelium. Both apical-to-basolateral and basolateral-to-apical directions can be modelled to predict the absorptive and efflux activity, respectively. [222]

The cell monolayer becomes confluent in 25-35 days, which can be followed by several methods. A simple and rapid technique is the measurement of transepithelial electrical resistance. [222] Electrodes are placed into the medium of both apical and basal chamber, and the resistance is easily determined by a voltohmmeter. The higher the TEER, the more complete the integrity. In general, the TEER increases and the paracellular permeability decreases with the culture time. TEER reaches a plateau value between the 25^{th} and 30^{th} days. The TEER of a confluent Caco-2 monolayer is not equivocal, it depends on several factors (e.g. passage number, the medium substances). Hidalgo *et al.* reported 174Ωxcm^2 , Artursson *et al.* reported 260Ωxcm^2 and 300Ωxcm^2, while Augustijns *et al.* determined the maximum at 700-900 Ωxcm^2. [223] In our work cell monolayer was used after reaching at least 1000Ωxcm^2.

The Caco-2 transport system is appropriate for both transcellular and paracellular transport modelling. Passive diffusion of small, hydrophilic molecules occurs across the intercellular tight junctions. Lucifer Yellow is a suitable marker of the paracellular pathway with easy

detectability. [222, 224] (Figure 14) The effect of a certain substance on the monolayer, thus tight junction integrity, can be evaluated by the comparison of LY transport in and without the presence of the examined substance.

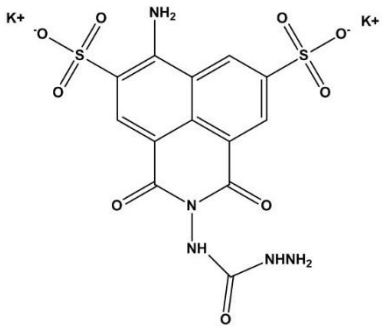


Figure 14 *Molecular structure of Lucifer Yellow.*

B. Hemolytic effect of cyclodextrins

For a long time, CDs have been considered to have two main drawbacks, namely: the not negligible renal and haemolytic toxicity. However, when these statements were maintained, mainly the parent CDs were available, and there was only a limited access to their derivatives. It is unfortunate though, that the main drawbacks limiting the practical application of CDs are the result of their greatest advantage, namely their potential to inclusion complex formation.

Ohtani *et al.* have already reported in 1989 the effects of parent CDs on human erythrocytes from various viewpoints. [225] It has been stated, that the haemolytic effect is in connection with the complexation of membrane components, thus the disruption of the membrane integrity. The type of extracted molecules depends on the CD cavity dimensions. β-CD is known for cholesterol extraction, meanwhile α-CD has greater affinity to phospholipids. The potency of cholesterol solubilizing followed the β>>γ order while α-CD had negligible effect, but for phospholipids the order was: α>β>>γ. [225] β-CD proved to be the most potent also in protein removal from the erythrocyte membrane. The erythrocytes' characteristic shape changes were observed prior to haemoglobin release in the presence of α- and γ-CD, though α-CD induced it at lower concentration: from biconcave discocyte through monoconcave stomatocyte then eventually to a round spherocyte. [225] β-CD caused direct swelling of the RBCs, leading to hemolysis. (Figure 15)

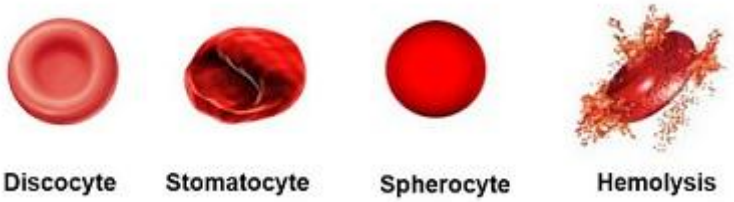


Figure 15 *Shape changes of human erythrocytes, induced by the presence of native α- and γ-CD. [226-229]*

The membrane disruptive effect of CDs is merely different from detergents. Detergents can build in the membrane bilayer, but after a certain concentration (CMC), they form micelles in the aqueous phase. The lipid components are extracted from the cellular surface into this structure. CDs do not build in the membrane lipid layer, but they form a lipid-containing compartment in the aqueous phase, where lipid components are extracted to. [225]

α-CD has high affinity towards phospholipids, but it is the highest towards phosphatidylinositol. The process of PI extraction builds up from two separate steps: the macrocycle first interacts with the polar inositol headgroup, and then complexes the acyl chain. [230] It was found, that those phospholipids, which have high affinity for α-CDs, are located in the internal leaflet of the erythrocyte membrane (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine). [231] Debouzy *et al.* suggested, that the modification of the global charge or the dipole moment of the α-CDs may result in decreased haemolytic activity. Namely, substitution of the hydroxyl rim with positively charged groups intends to decrease, while the overall negative charge increases the haemolytic character. [231] 6-peramino derivatives had practically no hemolytic effect, while per-6-O-methylated derivatives had increased hemolytic activity compared to the parent molecule. [231] These suggestions gave opportunity to exploit the advantages of α-CD derivatives in terms of pharmaceutical use, instead of stigmatizing them due to the haemolytic activity of the parent molecule.

Later, the haemolytic activity of several CD derivatives had been evaluated by several research groups. [181] Leroy-Lechat *et al.* reported the order of haemolytic activity on human and murine RBCs as follows: β-CD> α-CD > HPBCD > γ-CD >> HPGCD ≥ HPACD. [232] Irie and Uekama determined the sequence of DIMEB >> TRIMEB >> β-CD > 6-O-maltosyl β-CD ≥ HPBCD (DS=5.8) ≥ α-CD >>> γ-CD > DHPBCD (DS=5.9) which shows the undoubted importance of DS. [179] With regard to sulfobutylether β-CD derivatives, it was found that: β-CD > SBEBCD (DS=1) > SBEBCD (DS=4), and practically no haemolytic effect was observed in case of SBEBCD with DS=7. [181]

C. Toxicological profile of cyclodextrins in pharmaceutics

CD derivatives differ in their toxic effect on different cell types or epithelial structures. These differences are driven from the cavity dimensions, the type of substituents and also the barrier structure. The versatility of CD based pharmaceutics is shown in Table 3. [173]

Table 3 *CD use in pharmaceutical products in terms of administration types.*

	α-CD	β-CD	γ-CD	HPBCD	SBEBCD	RAMEB
Oral		+	+	+	+	
Rectal		+		+		
Parenteral	+			+	+	
Dermal		+	+	+		
Nasal						+
Ocular		+		+		+

Oral administration

The oral bioavailability of CDs is low, as they do not absorb from the GI tract only in negligible rate, due to their high hydrophilicity and bulky structure. [181] Their low oral bioavailability and the simultaneous ability to complex lipophilic BAMs make CDs an ideal solubility, thus bioavailability enhancer for BCS II and IV drugs. [173]

CDs have side effects only in case of high dose administration (>1000 mg/kg/day), namely reversible diarrhoea and cecal enlargement. However, these symptoms are typical when large amount of poorly digestible carbohydrates or other osmotically active nutrients were taken. [181]

Examples for CD-based oral formulations: [233]

- Zyrtec chewing tablet: cetirizine + β-CD
- Nicorette tablet: nicotine + β-CD
- Peridopril Erbumine tablet: perindopril tert-butylamine + HPBCD

Rectal administration

When CD containing suppositories are applied, CD can be detected in the urine, hence it absorbs from the rectum. It can be explained either by its effect on the permeability via rectal mucosa or the interactions between the CD and glycerides, as cardinal components in

suppositories. [173] High absorption rate was observed mostly in case of HPBCD and RAMEB. [234] There were no irritation effect up to 230mg of β-CD administration in human. On the contrary, α-CD potentially causes damage in the epithelial cell layer. [179]

Examples for CD-based rectal formulations: [176, 233]

- Prepulsid: cisapride + HPBCD
- Brexin: piroxicam + β-CD
- Mobitil: meloxicam + β-CD

Parenteral administration

The safety criteria for parenteral use are the most throughout and strict regarding the tolerance of human. α- and β-CD cannot be applied in parenteral formulations as they have severe renal toxicity. [173] Native β-CD have another drawback due to its lack of solubility. Interestingly, and despite of the renal effect, α-CD is used in a parenteral formulation with prostaglandin. Recently there is no marketed γ-CD containing parenteral product, however its toxicological profile is favourable. Only reversible vacuolization was observed in the renal tubular epithelium, which did not affect the renal functions. [173] β-CD derivatives (except for HPBCD and SBEBCD) seem to be more toxic than the parent molecule, thus their parenteral utilization is not recommended. [235]

HPBCD and SBEBCD are the most often used CDs in parenteral formulations. [196, 197] However, these two derivatives can result reversible vacuolation of the kidney tubular cells without affecting the kidney functions. Mostly long treatment causes this effect, which indicates the importance of the duration of exposure. This type of vacuolation can be observed when osmotic agents such as glucose, mannitol or dextran is excreted at high concentrations, thus it may be an adaptive response. [173]

Examples for CD-based parenteral formulations: [233]

- Prostandin 500: prostaglandin E1 + α-CD
- Dyolect: diclofenac + HPBCD
- Abilify: aripiprazole + SBEBCD

Dermal administration

CDs alone have no significant transdermal absorption. However, CDs can complex some dermal components, e.g. cholesterol or triglycerides, which results in reduced skin barrier

function and increased drug absorption. [173] Native CDs in high concentrations proved to cause irritation in the following order: α-CD < γ-CD < β-CD, which corresponds with the lipid complexation ability from the skin. [179] However, up to 0.1% these parent molecules can be safely used in dermal formulations. [173] HPBCD is accepted to be safe (based on antigenicity, mutagenicity and topical irritation tests) like those materials used in perfumes and cosmetics. [179]

Examples for CD-based dermal formulations: [233]

- Glymesason: dexamethasone + β-CD
- Pain relief gel: menthol/camphor + β-CD

Nasal administration

CDs in high dose are proven to enhance drug bioavailability via nasal mucosa. [173] The mechanism of their effect is in connection with the ability of mucosal lipid complexation, thus the reversible disruption of membrane integrity. [236] The nasal application of steroids has a predilection of CD usage, as the intestinal and hepatic first-pass effect can be avoided. Furthermore, when CDs are in complex form, they are not available for mucosal component complexation, thus the integrity of nasal mucosa stays intact. [179] HPBCD and RAMEB solutions up to 10% can be administered with no harmful effect on the mucosa, and β-CD is used under 1.5%.

CDs also improve the pulmonary delivery of drugs (e.g. insulin), although their intratracheal absorption is considerable. [179]

Example for CD-based nasal formulation: [176]

- Aerodiol: 17β-estradiol + RAMEB

Ocular administration

Due to the layered construction of the eye, the tight barrier function of the cornea, the small accessible volume and the continuous presence of eye water, ophthalmic drug delivery is challenging. CDs are advantageous as they may carry the lipophilic APIs through the aqueous mucin layer to the lipophilic eye surface. [237] α-CD can enhance drug transportation via the cornea, although toxic effects occur at concentrations higher than 4%. [173] RAMEB is safe to use under 5%, and SBEBCD and HPBCD did not cause irritation up to 10 and 12.5%, respectively. [173]

Example for CD-based ocular formulation: [233]

- Vitaseptol: thiomersal + β-CD
- Indocyllir: indomethacin + HPBCD
- Voltaren Ophtha: diclofenac + HPGCD

Calixarenes

Calixarenes are usually described as structures, providing practically unlimited possibilities due to the ease of their synthesis, the achievable selective modification at their different faces, and the ability of inclusion complex forming with guest molecules. Furthermore, their negligible hemolytic effect is also a notable advantage. [238]

V. History

The history of calixarenes reaches back to the end of the 19th century. Similarly to the past of CDs, it was interspersed with twists and turns and challenges.

The chemistry of aldehydes and phenol in the presence of strong acid was first examined by Adolph von Bayer [239], however in that time no accurate analytical methods were available to characterize the products, and formaldehyde was not easily available. In 1894, Lederer and Manasse studied the reaction of phenol and formaldehyde, but in basic conditions. They obtained *o*- and *p*-hydroxymethylphenol, as crystallized solids. [239] The big breakthrough which made the aldehyde-phenol reactions indeed significant, is bounded to Leo Hendrik Baekeland, who invented inter alia the bakelite. [240]

Phenol reacts at both the *ortho*- and *para*- positions, thus a highly cross-linked polymer results, in which one phenol residue attaches to three other. When one of these two positions is protected, the number of evolved connections is limited, resulting only chain-like oligomers. (Figure 16) [241] Alois Zinke performed studies using *p*-substituted phenols in their condensation reaction with formaldehyde. [242]

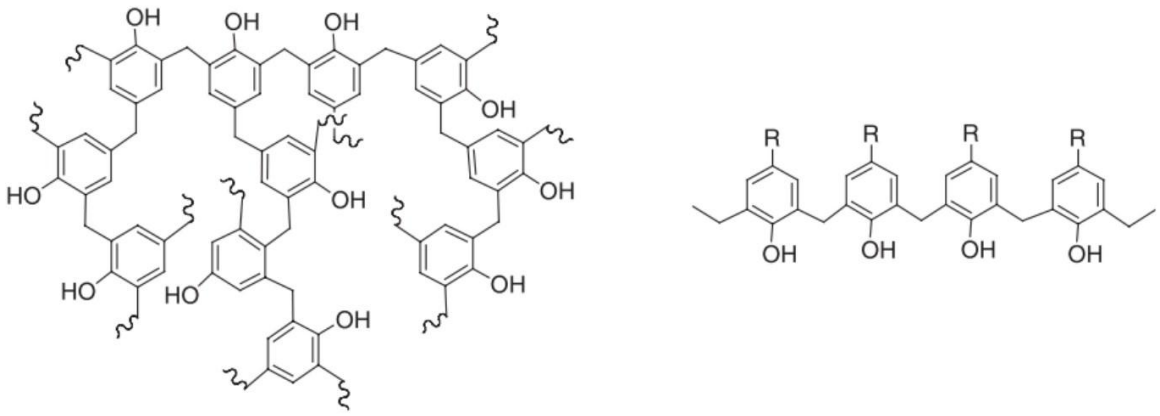


Figure 16 *Phenol reacts at para- and ortho-positions to form a cross-linked polymer, while para-substituted phenol reacts only at the ortho-position to give a linear product. Figure is modified from [239].*

The first solid experimental evidence to support the tetrameric structure of phenol- and resorcinol-aldehyde products was given by Joseph Niederl, on the base of molecular weight determinations. [239]

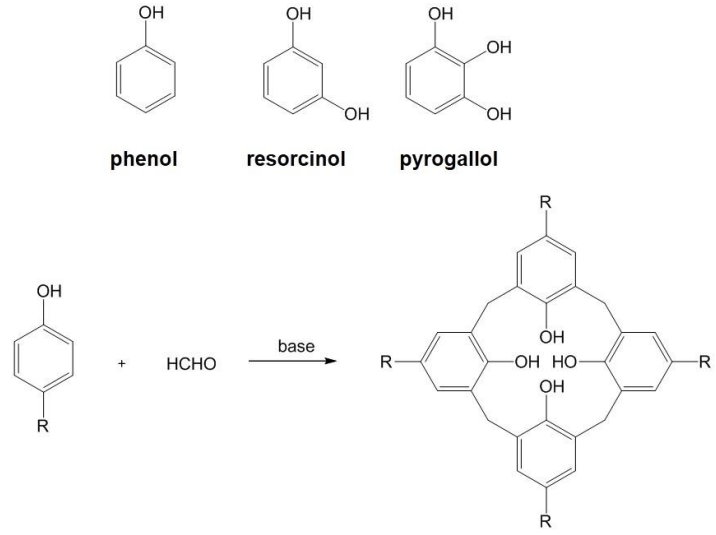
From the 1970's, the Zinke cyclic tetrols attracted much attention from the field of enzyme mimics. Those molecules, which contain cavities can be suitable 'molecular baskets'. CD usage had one drawback, namely their complicated isolation from natural sources, thus the utilization of Zinke cyclic tetramers proved to be the best option as they are accessible by relatively easy synthesis, and they are truly basket-like. [239]

The Zinke cyclic tetrols' non-planar character, thus their vase shape and their orientation similar to the Greek *calix crater* (Figure 17), lead to the *calixarene* name, which appeared from 1978, and later became accepted by the IUPAC as well. The Greek '*calix'* means vase, and '*arene'* refers to the aryl residues in the macrocyclic array. [243]

Figure 17 *Calixarenes are named after a Greek vase called calix crater due to their similar shape. Image is from [244].*

VI. Structure and nomenclature

Calixarenes are build up from 4 to 9 phenolic units linked by methylene groups at the *ortho*-position. The aromatic building residues are driven from phenol, resorcinol or pyrogallol and from their derivatives. (Scheme 3) By their covalent bonding, a three-dimensional basket or vase shaped macromolecule is formed. [239] The vase-like macrocycle is characterized by a wide upper rim, a narrow lower rim and the annulus in between. Calixarenes should be represented stand upright, with the hydroxyl groups pointing downwards (*endo*) and the *para*-substituents upwards (*exo*). (Figure 18) The hydroxyls on the lower rim form intermolecular hydrogen bonds, which stabilizes the conformation. [245]



Scheme 3 *The building units of calixarenes are the aromatic phenol, resorcinol, pyrogallol or their derivatives modified at the para- or meta-position.*

The name calixarene originally referred to Zinke's tetramer, although since then several derivatives were discovered consisted of more than 4 aryl groups. To accommodate the name of these macroolygomers, the number of the building units is bracketed between *calix* and *arene* (calix[n]arene). If there is, a *para*-substituent is designated by name, such as *p*-tert-butylcalix[4]arene. [239]

Calixarenes exist in several conformational forms as the rotation is allowed around the methylene bonds. The conformational flexibility is influenced by the size of the macrocycle. [243, 245] It was assumed, that the flexibility increases with the ring size, but Gutsche proved that it is not necessarily the case. Calix[4]arenes and calix[8]arenes show almost identical behaviour, but calix[5]arens, calix[6]arenes and calix[7]arenes act in a more expected fashion.

[245] For instance, calix[4]arene has four discrete forms called *cone*, *partial cone*, *1,2 alternate* and *1,3 alternate*. The presence of hydroxyl groups makes the strong intramolecular hydrogen bonding possible, which is mostly remarkable in case of calix[4]arenes and calix[5]arenes, thus they prefer the cone shape conformation. As the number of building units increase, the preferred conformation becomes planar. [245]

The calixarene cavity, similarly to CDs, is much more hydrophobic, as the annulus is formed by the aromatic frames. They possess different chemistries at the phenolic functions and at the *para*-positions, which facilitates the selective modification of the upper or lower rim. [239, 243]

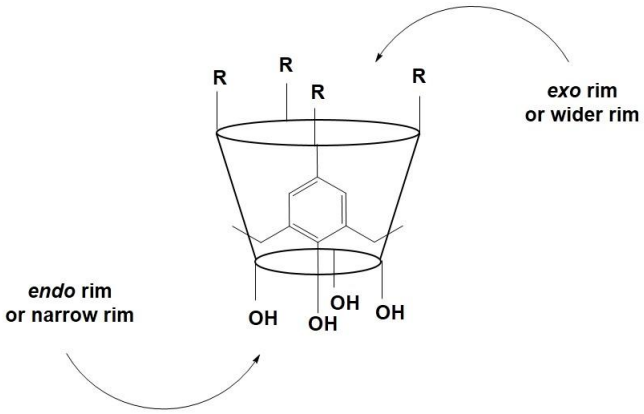


Figure 18 *Scheme of a calixarene macrocycle, which is often portrayed stand upright, with the wider rim pointing upwards and the narrow rim pointing downwards.*

VII. *Para*-sulphonato-calix[n]arenes

During the 1980's, a general demand arose for the production of water soluble calixarenes as the unmodified parent molecules possess low aqueous solubility. The main objective was to create artificial water soluble host compounds with hydrophobic cavity of defined size and shape, able to form inclusion complexes, and can be used as a synthetic analogue of CDs. [238] The first soluble derivatives were synthetized by Arduini using *para*-tert-butyl-calix[4]arene as a starting material, which was an interesting choice as it forms 1:1 or 2:1 inclusion complexes with aromatic guest molecules. [246] Though other calix[4]arenes gave clathrates or channels of the crystal lattice. [247-249] The first derivatives with satisfying aqueous solubility were the alkali and ammonium salts of *para*-tetra-butylcalix[4]arene tetracarboxylic acid, maintaining the cone structure and carboxylic groups on the wider rim. This product allowed the study of inclusion complex formation in aqueous environment in the first time, and its comparison with the results obtained in solid state. [246]

The first representative of water soluble calix[6]arenes were the *para*-sulphonato-calix[6]arenes, first synthetized in 1984. [250] (Figure 19)

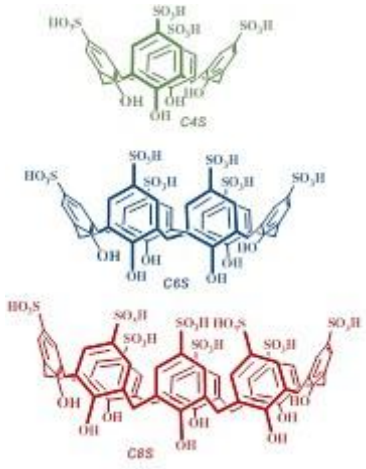


Figure 19 *Scheme of para-sulphonato-calix[n]arene. (n=4, 6, 8)*

The three main approaches, facilitating their production includes the direct *ipso*-sulfonation of *para*-tert-butyl-calix[n]arenes, the sulfonation of *para*-H-calix[n]arenes or chlorosulfonation. (Figure 20) The most common procedure is the direct sulfonation, however e.g. the solubility of the starting material can hinder its application. When substituents on the lower rim attend, chlorosulfonation is the method of choice. [250] Mono-functionalization on the narrow rim

with amino or carboxylic groups facilitates the amelioration of their interactions with biologically active molecules or their grafting onto solid surfaces. [238]

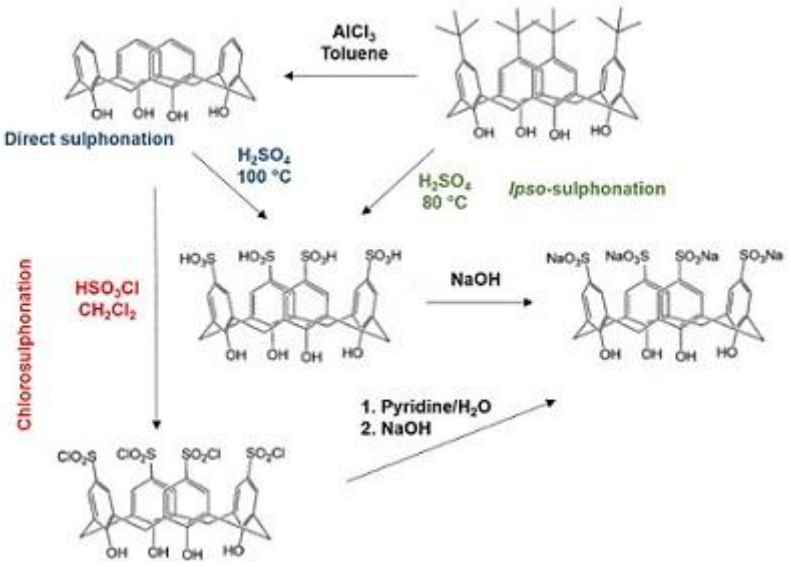


Figure 20 *Pathways of para-sulphonato-calix[4]arene synthesis. Figure is modified from [238].*

Biological activity

The fact, that several water soluble *para*-sulphonato-calix[n]arene derivatives proved to have biological activity, gives them a remarkable importance. This notable relevance is partially driven from their high affinity to particular amino-acids. For instance, it results their antithrombotic activity, enzyme inhibition or their role in prion diagnosis. [238]

Drug development recently transforms its philosophy from ‘one drug one target’ to ‘one drug multiple targets’. Polypharmacology involves the discovery of a single BAM with several targets, thus unknown off targets of already applied APIs may be discovered. [251] There is a great possibility that *para*-sulphonato-calix[n]arenes can function as multi-targeting compounds.

Antituberculous activity

The first paper related to the biological activity of calixarenes is from 1955. Cornforth *et al.* reported the *in vivo* antituberculous activity of *para*-octyl-calix[8]arenes, maintaining polyoxyethylene units on the lower rim. [252] Interestingly, no correspondence was found between the *in vitro* ability to disperse *Mycobacterium tuberculosis* and the *in vivo* chemotherapeutic effect to suppress acute tuberculosis on mice. [252]

Anti-thrombotic activity

The presence of macrocycles can prolong the coagulation time. [238] Due to their flexible structure and the presence of several sulfate groups, *para*-sulphonato-calix[n]arenes show structural analogies with heparin, one of the most important anticoagulant therapeutic agent. [253] The main amino-acids present in the heparin recognition peptide sequence are lysine and arginine, and the complexation ability of *para*-sulphonato-calix[n]arenes towards these amino-acids was widely studied. [254] The introduction of a carboxylic function to the narrow rim proved to enhance the anticoagulant activity. Not only the substituent types, but the size of the macrocyclic ring seems to play role in the anticoagulant activity. As the number of building units increase, the anticoagulant property escalades. It has been shown that their mechanism is based on the interaction with serine protease inhibitors, such as HC II and AT. [255]

Enzyme inhibition

Para-sulphonato-calix[n]arenes can play role in enzyme inhibition too, as it was proved in case of L-lysil oxidase. [238] The electrostatic interaction formed between the lateral chains of the basic amino-acids of the enzyme's active site and the sulfonate groups of calixarene, hinders the normal function of the L-lysil oxidase, which is responsible for the covalent binding of extra-cellular macromolecules and the initiated reticulation. By this action a new way of fibrinolytic disease treatment was introduced, namely in the therapy of skin cicatrisation. [256]

Ion channel inhibition

The first effect related to ion channels was published by Atwood *et al.*, in which *para*-sulphonato-calix[n]arenes were shown to have influence on chloride dependent ion channels. More precisely, the inhibitory effect increased with the macrocycle size. [257]

Later, great effort was devoted to study the *para*-sulphonato-calix[n]arenes' and their derivatives' impact on volume-regulated anionic channels. These channels mostly take place in the endothelium, and they have important role in cell volume and intracellular ionic strength regulation. [258] The influence of calixarenes depends on the size of the ring and the membrane potential. The 4 units calixarenes occluded the VRACs but only at positive potential and did not have effect on negative potential. Furthermore, when the potential was highly positive, calixarenes passed through the channels. The 6 and 8 units calixarenes have the advantage of not permeating through the channel neither on remarkable positive potential, but their inhibitor effect on moderate potentials is less considerable. [259]

Antiviral activity

Those calix[n]arenes, which possess polar substituents with terminal carboxylate, phosphate or sulfate group, are able to interact with virus envelopes (such as HIV or Herpes) driven by electrostatic interactions. Due to this effect, the recognition site of the virus is masked, thus the cell infection is hampered. [260, 261]

Antibiotic activity

By the tested 57 water soluble calix[n]arene derivatives, 7 (including *para*-sulphonato-calix[n]arenes) proved to have antimicrobial activity against *Corynebacterium*. [262] As further studied, these *para*-sulphonato derivatives were found to maintain anti-microbial effects against *Fusarium solani f. sp. Mori*., *Rosellinia necatrix* and *Colletotrichum dematium*. [262]

Prion diagnosis

Structural changes in the normal amino-acid sequence of PrP^{C} can lead to the formation of pathogenic PrP^{SC}, which is the origin of diseases such as scrapie in sheep, bovine spongiform encephalopathy in cattle or Creutzfeldt-Jakob disease in humans. [263] These proteins generally maintain a negatively charged surface and a positively charged pocket area which is rich in lysine and arginine. As previously detailed, calixarenes, especially *para*-sulphonato-calix[n]arenes have high affinity to these amino-acids, thus they tend to be potential markers in prion disease detection. Mono-substituted derivatives proved to be more effective than the non-substituted parent molecules. [238]

Toxicity

The first *in vitro* test performed on calixarenes was done by Gutsche, who reported that they had no activity in the Ames test, thus calixarenes appeared to have no mutagenic effect on the tested bacteria strain. [264] Later, phosphate derivatives were tested and proved to have no effect on cell growth of human fibroblasts. [238] Regarding the activation and cell viability tests performed on nucleophile granulocytes with *para*-sulphonato-calix[n]arenes, similar results were obtained, namely no non-specific immune response was triggered, and neither cell number reduction was observed. [265] If there is, one of the main advantage of calixarenes over CDs is their negligible hemolytic activity. *Para*-sulphonato-calix[n]arenes and their mono-substituted derivatives on the lower rim were tested. Up to 50mM concentration, less than 5% of hemolysis was observed in case of each tested derivatives. However, it has to be mentioned that the hemolytic effect increases with the macrocycle ring's size. [266]

Drug solubilisation

However, calixarenes are not yet approved by the FDA for their use as pharmaceutical excipients, their structural similarity to CDs generated interest in their future application as solubilizing agents. Yang *et al.* performed a notable work focusing on practically insoluble drug molecules, such as furosemide [267], nifedipine [268] or niclosamide [269], and their inclusion complex formation with *para*-sulphonato-calix[n]arenes. The results are promising, calixarenes undoubtedly enhanced the solubility of the above mentioned compounds. However, in case of niclosamide, the effect of HPBCD could not be outperformed, but by combining HPBCD and *para*-sulphonato-calix[6]arene, the solubilizing effects were additive. [269]

Experimental part

VIII. Scheme of work

Biocompatibility evaluation of α-cyclodextrin derivatives

Our research was inspired by the scarce data found on the biocompatibility evaluation of α-CD derivatives. The lack of research has been focused on the description of their toxicological profile results in the poor understanding of structure-activity relations. Driven by the devotion for the better understanding, those of the modified α-CDs which are available on the market, were tested and ranked based on their toxicity. (Table 4) MTT cell viability test and RT-CES method was used to define the IC_{50} values on Caco-2 cells. Hemolysis test was performed on human erythrocytes, and the HC_{50} values were determined.

Table 4 *The most frequent derivatives are classified as esters, ethers and deoxy CDs, furthermore glucopyranose-ring modified and deuterated CDs also exist. [59] This table represents the examined α-CD derivatives, produced by Cyclolab Ltd.*

	α-CD derivative	Short name	Molecular formula	Molecular weight	DS
	native		$C_{36}H_{60}O_{30}$	972.84	0
ethers	partially methylated	PARMEA	$C_{45}H_{78}O_{30}$	1099.1	~9
ethers	random methylated	RAMEA	$C_{47}H_{82}O_{30}$	1126.9	~11
ethers	2,3-tri-O-methyl	TRIMEA	$C_{54}H_{96}O_{30}$	1225.4	18
ethers	(2-hydroxy)propyl	HPACD	$C_{49.5}H_{87}O_{34.5}$	1234.3	~4.5
ethers	carboxymethylated Na-salt	CMACD	$C_{48}H_{63}O_{36}Na_3$	1212.9	~3.5
esters	sulfated Na-salt	sulfated	$C_{36}H_{48}O_{66}S_{12}Na_{12}$	2197.4	~12
esters	phosphated Na-salt	phosphated	$C_{36}H_{60}O_{42}P_4Na_4$	1380.7	~2-6
esters	succinyl	SuACD	$C_{52}H_{76}O_{42}$	1373.2	~4
esters	acetylated	AcACD	$C_{52}H_{76}O_{38}$	1267.1	~7
polymer	carboxymethyl-α-CD crosslinked with epichlorohydrin	CMACDEp		55kDa	

Synthesis of α-cyclodextrin derivatives

Most of the commercially available CDs are known to be defined only by approximate DS, thus within a batch, different structures represent. (Table 4) Our aim was to investigate the effect of well characterized structural changes on the toxic behaviour. We planned to carry out

individual primary and secondary face per-substitution, and complete per-substitution. (Figure 21) The synthetized derivatives were alkyl ethers, bar none. Regarding the substituent types methyl-, ethyl-, propyl- and butyl chains were introduced. However, the increasing length of the alkyl chains at the secondary phase caused decreasing aqueous solubility. Further modifications were performed on the free OH-groups at the C-6 position. Namely, negatively charged groups were substituted to increase the hydrophilicity. The presence of a sulfate or sulfopropyl group significantly increased their solubility.

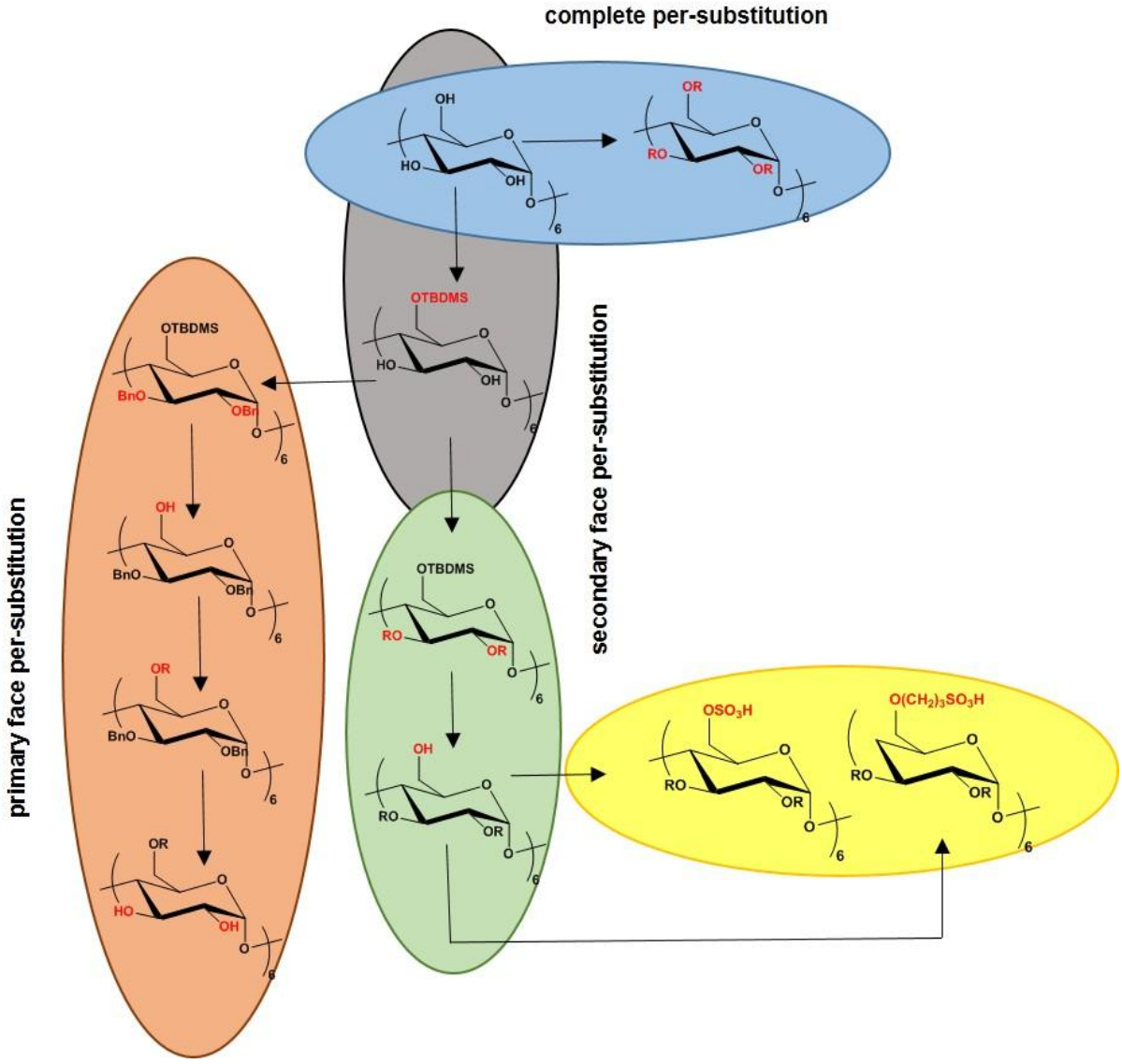


Figure 21 *Scheme of α-CD substitution.*

Transport study of para-sulphonato-calix[n]arenes

Para-sulphonato-calix[n]arenes are the result of an endeavor aiming the solubility enhancement of calix[n]arenes. The introduction of sulfate groups at the para position resulted in the first highly water soluble derivatives. From this moment, calixarenes become suitable for enhancing the solubility of guest molecules. Our aim was to explore the effect of three different *para*-sulphonato-calix[n]arenes on the paracellular transport through Caco-2 cell monolayer. First, the IC_{50} values were individually defined on Caco-2 cells by MTT assay. It allowed to determine a concentration range where the possibility of the monolayer disruption, thus the potential elevation of absorption rate resulted by a non-confluent monolayer can be excluded. In the transport study, LY was used as the marker of paracellular absorption. LY transport was modelled in the simultaneous presence of calix[n]arenes, and after a 30min pre-treatment with the macrocycles.

Contributions

RT-CES experiments were performed with the help of Dr. Alexandra Bocsik and were observed by Dr. Mária Deli. The comparison of the toxicity of RAMEA and RAMEA-phosphatidylcholine complex was done with Dr. Zoltán Ujhelyi. Dr. Rudolf Gesztelyi did the statistical analysis. Dr. Ildikó Bácskay observed and helped in the evaluation of biocompatibility assays. Dr. Florent Perret monitored the evaluation of the NMR spectra. Dr. Anthony W. Coleman evaluated the results of transport study on calixarenes.
The rest of the experimental work including cell viability assays, hemolysis tests, transport study, and α-CD synthesis have been done by the author.

IX. Materials

α-cyclodextrin derivatives

The commercially available α-CD derivatives were generously offered by Cyclolab Ltd. (Budapest, Hungary), namely:

- randomly methylated α-CD,
- hexakis-(2,3,6-tri-O-methyl)-α-CD,
- partially methylated- α-CD
- (2-hydroxy)-propyl α-CD,
- sulfated α-CD sodium salt,
- phosphated α-CD sodium salt,
- carboxymethylated α-CD sodium salt,
- succinylated α-CD,
- acetylated α-CD,
- carboxymethylated α-CD polymer crosslinked with epichlorohydrin.

RAMEA:phosphatidylcholine complex was also synthetized and provided by Cyclolab Ltd.

Compound **12**, **13** and DIMEA (compound **7**) were synthetized as described below.

We investigated each α-CD derivative in 7 different concentrations: 0.01; 0.1; 1; 10; 25; 50 and 100mM, apart from TRIMEA, which was not soluble above 30mM and DIMEA above 75mM. The highest concentration tested of compound **12** and **13** was 50mM, due to the small amount of available products. The solid samples were dissolved in isotonic phosphate buffered saline (PBS, pH=7.2), purchased from Sigma–Aldrich (Budapest, Hungary).

Calixarenes

C4S, C6S and C8S were synthetized by Florent Perret and Anthony W. Coleman as described in the literature. [250]

Caco-2 cell line

Caco-2 cell line was obtained from the European Collection of Cell Cultures. Cells were grown in plastic cell culture flasks in Dulbecco's Modified Eagle's Medium, supplemented with 3.7g/L $NaHCO_3$, 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) non-essential amino acids

solution, 1% (v/v) L-glutamine, 100 IU/mL penicillin, and 100µg/mL streptomycin at 37ºC in an atmosphere of 5% CO_2. The cells were routinely maintained by regular passaging. For cytotoxic experiments, cells were used between passage numbers 20 and 40. The culture media was replaced with fresh media in every 72 hours.

Reagents used for synthesis

All reagents were obtained from commercial sources and used as received. Reagents and methanol were purchased from Sigma Aldrich, the other solvents from VWR. The use of anhydrous solvents was provided by PureSolv solvent purification system by Innovative Technology. Anhydrous THF was freshly distilled in the presence of Na and benzophenone. All reactions were carried out in N_2 atmosphere with the exception of hydrogenation reactions where H_2 was used.

Analysis

^1H (300Hz and 400Hz) and ^{13}C (100Hz) NMR spectra were recorded on Bruker AV 300 and AV 400 instruments. The assignment of ^1H and ^{13}C was supported by one-dimensional DEPT and two-dimensional ^1H-^1H COSY experiments. Experiments were recorded by using $CDCl_3$, MeOD or D_2O as solvent. 5-17mg of sample was directly dissolved into the NMR tube in 0.75ml of solvent. Chemical shifts (δ) and coupling constants (J) are given in ppm and Hz, respectively. The peaks patterns are indicated as the following format multiplicity (s: singlet; d: doublet; t: triplet; m: multiplet; dd: doublet of doublet).

High Resolution Mass Spectra were recorded on SYNAPT G2 HDMS (Waters), on QStar Elite (Applied Biosystems SGIEX), on MicrOTOFQ II (Bruker) equipped with an Atmospheric Pressure Ionization (API) source or on a Time Of Flight (TOF) analyser. Mass spectra was recorded in a positive ion mode for most of the derivatives, except for the anionic sulfonate CDs, for which negative ion mode was used. Samples were prepared in 2mg/mL concentration.

Melting point determinations were performed on Büchi B-540 apparatus and are uncorrected.

Purification

Thin layer chromatography (TLC) was performed on normal phase (pre-coated TLC-sheets ALUGRAM® Xtra SIL G/UV254, by Macherey-Nagel) or reversed phase sheets (PLC RP-18 F254S, by Merck), and the chromatograms were developed by using 8% H_2SO_4 in EtOH and dried at 180°C.

Purification steps included normal and reverse phase flash column chromatography. Normal phase column chromatography was performed either manually (technical grade silica, pore size 600Å, 230-400 mesh particle size, 40-63μm particle size, by Sigma Aldrich) or automatically by using CombiFlash® Rf200i (Serlabo Technologies) preparative chromatographic system. C-18 column chromatography was performed only on CombiFlash® system, applying MeOH-H_2O as an eluent.

Dialysis was performed by using Pur-A-Lyzer™ Mega Dialysis Kit (15-20mL, MWCO=1kDA) purchased from Sigma Aldrich. Sample volume was adjusted to 15-20mL, and 100-fold volume of pure solvent was used in the outer chamber, changed 3 times in every 2 hours. The applied solvent was Millipore Milli-Q Ultrapure Water.

Celite® 545 (Sigma Aldrich) as a filtrating agent was used for removing 10%Pd/C catalyst.

X. Methods

A. Hemolysis test

Hemolysis test was performed on fresh human blood, collected from healthy donors. Erythrocytes were separated from citrated blood by centrifugation at 2500 × g for 10min, washed three times with PBS and re-suspended in the same solution. Aliquots of the cell suspension with the respective RBC number of 5×10^7 were added to the buffer solution (PBS, pH 7.2) containing increasing concentrations of the samples investigated in the study. After mixing them gently, each solution was incubated at 37°C for 10 minutes and then centrifuged at 5000 × g. Finally, the absorbance of the hemoglobin released into the supernatant was measured at 540nm with FLUOstar OPTIMA Microplate Reader (BMG Labtech, Germany). The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of the sample solutions, related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water. The dose–response curve was determined, and the HC_{50} concentrations were subsequently calculated.

B. MTT cell viability assay

MTT assay was performed on Caco-2 cells for the evaluation of IC_{50} concentration for both CDs and calixarenes. The cells were seeded in 96-well plate until the cell monolayer become confluent. The medium was changed once. After one week, the medium was removed, the cells were washed with PBS and exposed to increasing concentrations of the certain substance dissolved in PBS. Cells were incubated for 30 minutes on 37ºC. Control groups were processed equally but incubated without CD. After the treatment, MTT dye (5mg/mL) was applied to each well for 3 hours. MTT solution was removed and isopropanol:hydrochloride acid (25:1) was added to dissolve the formazan crystals. The absorbance was measured at 570nm against a 690nm reference with FLUOstar OPTIMA Microplate Reader. Cell viability was expressed as the percentage of untreated control.

C. Real-Time Cell Electronic Sensing

The E-plate was coated with 0.2% rat tail collagen–DW solution for 20 minutes at 37°C. Culture media (60µL) was added to each well for background readings than 100µL Caco-2 cell suspension was dispensed at the density of 1.5×10^4 cells/well. The cells were grown for 2 days. The medium was changed to excipients solutions and the cells in the E-plate were kept in an incubator at 37°C for 8 hours and monitored every 5min. The CI at each time point was defined

as $(R_n–R_b)/15$, where R_n is the cell–electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the media alone.

D. Transport study

Firstly, the concentrations of the *para*-sulphonato-calix[n]arenes were set below the IC_{50} values. The used concentrations were: C4S=2.5mM, C6S=0.5mM and C8S=1.25mM.

Caco-2 cells were seeded in 12-well Transwell inserts (Corning Costar, USA) at a density of $2x10^5$ cells per cm^2, and was let to grow for 30-35 days. The TEER was measured when the medium was changed every third day (Millicell-ERS voltohmmeter, Millipore, USA). Tests were started, when the TEER values reached $1000\Omega xcm^2$. The culture medium was removed and the apical and basolateral compartments were washed with Hank's Balanced Salt Solution (purchased from Sigma Aldrich, Hungary).

In the experiments involving pre-treatment with calixarenes, the cells were exposed to 500µl of calixarene solution dissolved in HBSS, and applied in the apical chamber. HBSS solution (1.5mL) was placed in the basal chamber. The pre-treatment was carried out for 30min at 37°C. After treatment cells were washed with HBSS solution and then Lucifer Yellow (Sigma Aldrich, Hungary) dye was applied at 40µg/mL concentration diluted with HBSS into the apical chamber, and HBSS into the basal chamber.

In each case, samples were taken from the basal chamber in the 5, 10, 20, 30, 40, 50, 60, 90 and 120 minutes and were replaced by fresh HBSS. Samples were collected in 96-well black plate and fluorescence was measured at an excitation wavelength of 450nm by FLUOstar OPTIMA Microplate Reader. The TEER was measured before and after the experiment.

E. Statistical analysis

All data were obtained from three to five independent biological replicates and in the same experiments, four parallel concentrations (wells) were measured, unless otherwise stated. Raw data of cell viability and hemolysis were compared with one-way ANOVA (using Geisser-Greenhouse correction) followed by Tukey post-testing (because all data passed the D'Agostino and Pearson omnibus normality test). For this purpose, data related to five concentrations were selected. Statistical significance for the difference of means was assigned into one of five categories: $p > 0.05$ (not significant), $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$. (Tables 5 and 6)

Data presented as mean ± SD. Statistical analysis was performed with GraphPad Prism 6.05, while other calculations were made by Microsoft Office Excel 2013.

Table 5 *Comparison of cell viability values among the groups treated with different α-CD derivatives (the number of asterisks indicates the level of statistical significance, p < 0.005 (*), p < 0.001 (**), p < 0.001 (***), p < 0.0001 (****).*

Logarithm of Concentrations	−4	−3	−2	−1602	−1301
HPACD *vs.* Phosphated	**				
HPACD *vs.* Polymer	*				
HPACD *vs.* SuACD			*		
Sulphated *vs.* Phosphated	**			****	****
Sulphated *vs.* SuACD				****	****
Phosphated *vs.* AcACD	**				
Phosphated *vs.* CMACD			**	***	****
Phosphated *vs.* SuACD			***		
Phosphated *vs.* Polymer			*	**	****
AcACD *vs.* Polymer	*				
RAMEA *vs.* TRIMEA		*	****	****	
RAMEA *vs.* HPACD		*	***	***	**
RAMEA *vs.* Sulphated		*	***	**	
RAMEA *vs.* AcACD		**	****	****	**
RAMEA *vs.* Phosphatidylcholine + RAMEA		**	****	***	**
RAMEA *vs.* Phosphated			****	****	****
RAMEA *vs.* SuACD				****	****
RAMEA *vs.* Polymer				*	
native *vs.* RAMEA			**	***	****
native *vs.* TRIMEA			****	****	
native *vs.* Phosphated				*	
native *vs.* SuACD				***	
native *vs.* Sulphated					**
native *vs.* CMACD					**
native *vs.* Polymer					*
TRIMEA *vs.* Sulphated			****	****	
TRIMEA *vs.* Phosphated			***		
TRIMEA *vs.* CMACD			****	****	
TRIMEA *vs.* SuACD			****		
TRIMEA *vs.* Polymer			****	****	
SuACD *vs.* AcACD			*		
SuACD *vs.* Polymer				****	****
CMACD *vs.* SuACD				****	****

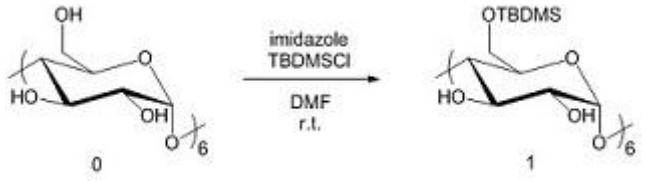
Table 6 *Comparison of hemolysis values among the groups treated with different α-CD derivatives (the number of asterisks indicates the level of statistical significance. p < 0.005 (*), p < 0.001 (**), p < 0.001 (***), p<0.0001 (****).*

Logarithm of Concentration	−2	−1602	−1301	−1
native *vs.* RAMEA	*		**	**
native *vs.* TRIMEA	****		****	****
native *vs.* HPACD	****	****	****	****
native *vs.* Sulfated	**	****	****	****
native *vs.* Phosphated		****	****	****
native *vs.* CMACD		****	****	****
native *vs.* SuACD	****	****	****	****
native *vs.* AcACD	**	****	****	****
native *vs.* CMACDEp	***	****	****	****
RAMEA *vs.* TRIMEA	****	*	****	****
RAMEA *vs.* HPACD	****	****	****	****
RAMEA *vs.* Sulfated	****	****	****	****
RAMEA *vs.* Phosphated		****	****	****
RAMEA *vs.* CMACD		****	****	****
RAMEA *vs.* SuACD	****	****	****	****
RAMEA *vs.* AcACD	****	****	****	****
RAMEA *vs.* CMACDEp	****	****	****	****
TRIMEA *vs.* HPACD	****	****	****	****
TRIMEA *vs.* Sulfated	****	****	****	****
TRIMEA *vs.* Phosphated	****	****	****	****
TRIMEA *vs.* CMACD	****	****	****	****
TRIMEA *vs.* SuACD	****	****	****	****
TRIMEA *vs.* AcACD	****	****	****	****
TRIMEA *vs.* CMACDEp	****	****	****	**
HPACD *vs.* Phosphated	****			
HPACD *vs.* CMACD	****			
HPACD *vs.* SuACD	****	****	****	***
HPACD *vs.* AcACD				*
HPACD *vs.* CMACDEp		****	****	****
Sulfated *vs.* Phosphated	**			
Sulfated *vs.* CMACD	***			
Sulfated *vs.* SuACD	****	****	****	*
Sulfated *vs.* CMACDEp		****	****	****
Phosphated *vs.* SuACD	****	****	****	
Phosphated *vs.* AcACD	**			
Phosphated *vs.* CMACDEp	****	****	****	****
CMACD *vs.* SuACD	****	****	****	
CMACD *vs.* AcACD	**			
CMACD *vs.* CMACDEp	****	****	****	****
SuACD *vs.* AcACD	****	****	****	
SuACD *vs.* CMACDEp	****		****	****
AcACD *vs.* CMACDEp		****	****	****

F. Synthesis of α-cyclodextrin derivatives

1. General approaches

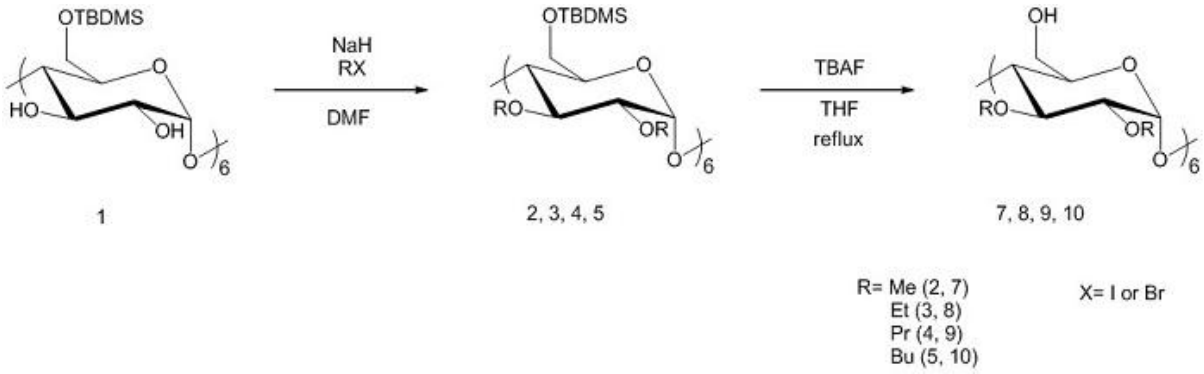
The starting material in each synthesis route was native α-CD (**0**). The key intermediate used in primary and secondary per-substitution was the hexakis (6-O-tert-butyldimethylsilyl)-α-CD (**1**). The regioselective protection of the C-6 hydroxyl groups was easily achieved with TBDMSCl in DMF, in the presence of a weak base, such as imidazole. (Scheme 4) The per-6-substituted product could be conveniently separated by flash column chromatography.



Scheme 4 *Scheme of 6-O-tert-butyldimethylsilyl α-CD synthesis.*

C-2 and C-3 per-substitution

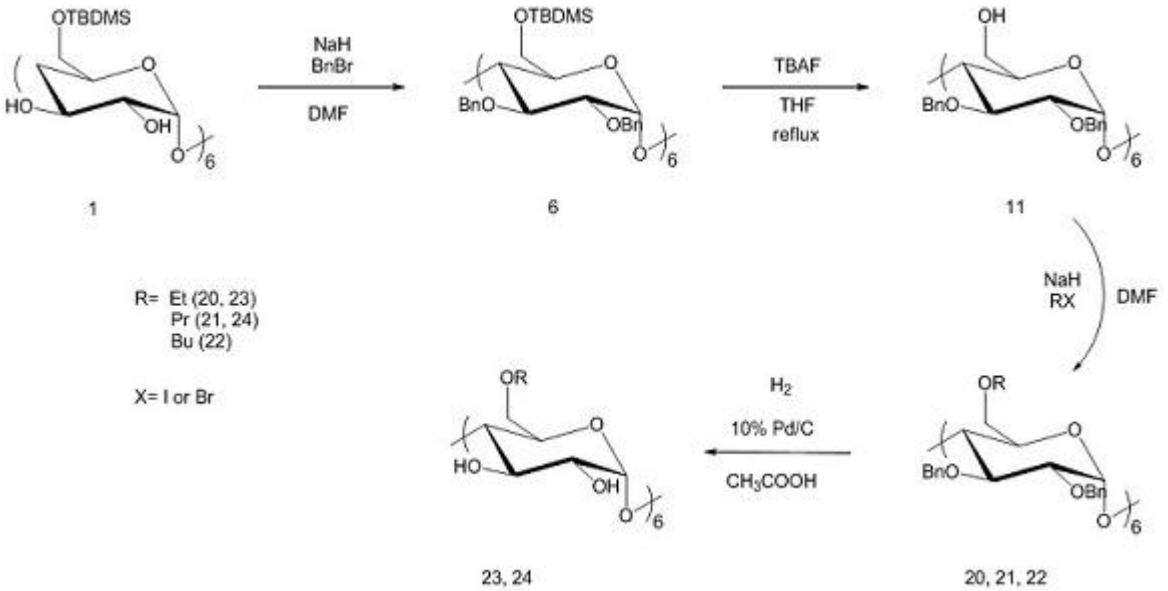
Compound **1** was used inter alia for the simultaneous substitution of C-2 and C-3 positioned hydroxyl groups, herewith avoiding the undesirable modification of the primary hydroxyls. The alkylation on the secondary face was achieved via another intermediate (2,3-dialkyl-6-O-TBDMS α-CD). Compound **1** was reacted in DMF with alkyl halides (most preferably iodides, otherwise bromides) in the presence of a strong base, such as NaH. [66] (Scheme 5) The increasing number of carbon atoms in the alkylating reagent requires consecutive alkylation steps, and by elevating the temperature more satisfactory yield can be achieved. [59] Nonetheless, the presence of under-substituted by-products was always inevitable, thus the main product was separated and purified with column chromatography. Pure compound **2**, **3**, **4** and **5** was desilylated, thus TBDMS group was exchanged for a proton. The reaction was performed in THF, in the presence of a TBAF, resulting (2,3-di-O-alkyl)-α-CDs. The crude product was purified with flash column chromatography to obtain compound **7**, **8**, **9** and **10**.



Scheme 5 *Scheme of per-alkylation on the secondary face via (2,3-dialkyl-6-O-TBDMS) α-CD intermediate.*

C-6 per-substitution

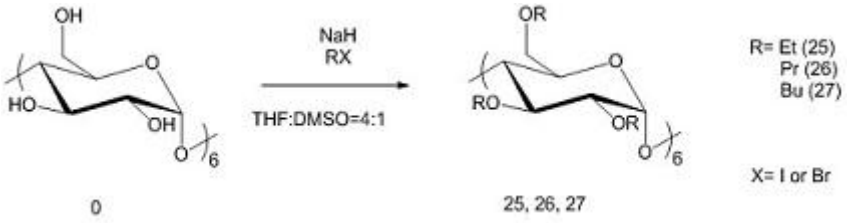
C-6 substitution was achieved via the 'long' way which consists of five steps. (Scheme 6) It started with the selective protection of the secondary face, after the liberation and alkylation of the C-6 hydroxyls, and the final step was the deprotection of the secondary face. Protection of compound **1** at C-2 and C-3 positions led to the (2,3-di-O-benzyl-6-O-TBDMS)-α-CD intermediate (**6**). Benzylation was attained in DMF by using BnBr in the presence of NaH. Compound **6** was deprotected at the C-6 positions by removing the TBDMS group with TBAF in THF. [66] (2,3-di-O-benzyl)-α-CD (**11**) was separated with flash column chromatography, then the C-6 hydroxyl groups were alkylated with the corresponding alkyl halide in the presence of NaH in DMF, meanwhile the secondary positions were still protected to avoid their alkylation (**20**, **21**, **22**). The last step was the removal of benzyl groups by hydrogenation in the presence of 10% Pd/C in acetic acid, obtaining the (6-O-alkyl)-α-CD derivatives (**23**, **24**).



Scheme 6 *Scheme of selective per-6-substitution.*

Complete per-substitution

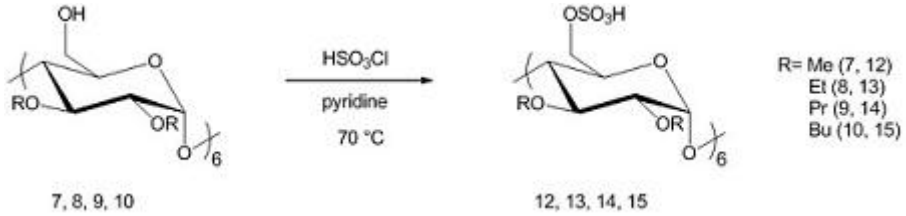
Complete per-substitution was achieved by using the 'sledgehammer' method, when the starting material was reacted with the alkyl halide in elevated equivalents. Dried native α-CD was used as a starting material. It was reacted in 4/1 THF:DMSO with an appropriate alkyl halide, in the presence of NaH. (Scheme 7) The crude product was purified with flash column chromatography.



Scheme 7 *Scheme of complete per-alkylation.*

(*2,3-di-O-alkyl-6-O-sulfate) α-CD derivative formation*

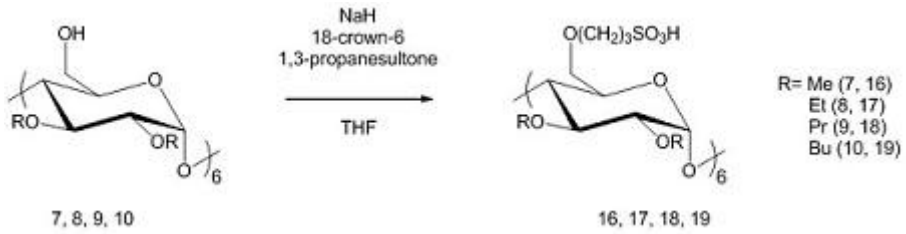
The reaction of compound **7**, **8**, **9** and **10** with chlorosulfonic acid in pyridine resulted 6-O-sulfate esters (**12**, **13**, **14**, and **15)**. [270] (Scheme 8) Methods used for eliminating the pyridine contained reverse phase column chromatography, filtration via celite, dialysis, and precipitation.



Scheme 8 *Formation of 6-O-sulfonates with chlorosulfonic acid.*

(2,3-di-O-alkyl-6-O-sulfopropyl) α-CD derivative formation

Sulfoalkylated α-CD derivatives were synthetized from compound **7, 8, 9** and **10** with using THF, NaH as a base, and 1,3-propanesultone. (Scheme 9) The reactivity was promoted by 18-crown-6 ether following the work of Kirschner *et al.* [271, 272] 18-crown-6 ether has high affinity to complex potassium and sodium especially in THF.



Scheme 9 *Synthesis of anionic derivatives from 2,3-dialkyl α-CDs in the presence of crown-ether.*

2. Synthesis protocols

Hexakis (6-O-tert-butyldimethylsilyl)-α-cyclodextrin (1)

Native α-CD and all used glassware were dried overnight at 60°C at low pressure for the total exclusion of moisture.

The dry α-CD (5.14mmol, 5g) and crystallized imidazole (66.87mmol, 4.55g, 13eq) were dissolved in 100mL of anhydrous DMF. After the total dissolution, TBDMSCl (36mmol, 5.42g, 7eq dissolved in 25 ml anhydrous DMF) was added dropwise during 15min under N_2 gas. The reaction was left running overnight at room temperature. After the complete disappearance of the starting material, the mixture was poured into cold water. The white precipitate was filtered through glass filter (No. 4), and washed with water. After it was dissolved in $CHCl_3$, and washed successively with 1M HCl, aqueous $NaHCO_3$ and water. The organic phase was dried on Na_2SO_4 and then concentrated. The solid was subjected to column chromatography ($CHCl_3$:MeOH:water = 50:10:1) to separate compound **1**.

Hexakis (2,3-di-O-methyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (2)

Compound **1** (1.8mmol, 3.0g) was dissolved in 150mL of anhydrous DMF and cooled to 0°C. NaH (60% in mineral oil, 72.4mmol, 1.74g, 40eq) was added to the solution and stirred for 1h at 0°C. MeI (90.5mmol, 5.7mL, 50eq) was then added dropwise and the reaction mixture was stirred at room temperature overnight. The same amount of NaH and MeI was added after 12 hours and stirred for another night. When TLC showed no presence of starting material, MeOH (20 mL) was added to the cooled reaction mixture to decompose the unreacted NaH. 100 mL of water was added, then extracted with diethyl ether. The organic layer was subjected to column chromatography (cyclohexane:EtOAc = 7:3) to obtain a pure white solid.

Hexakis (2,3-di-O-ethyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (3)

Compound **1** (0.3mmol, 0.5g) was dissolved in 20mL of 15/5 THF:DMF mixture and cooled to 0°C. NaH (60% in mineral oil, 10.85mmol, 0.26g, 36eq) previously washed with hexane was added, and the mixture was stirred for 1 hour. EtI (12.6mmol, 1.0mL, 42eq) was added dropwise and then the reaction was left overnight. The same amount of NaH and EtI was used after 24 and 48 hours. MeOH was added, then the reaction mixture was concentrated. The residue was dissolved in DCM and washed successively with water, aqueous $Na_2S_2O_3$ and water again. The organic phase was dried on Na_2SO_4 then evaporated. The yellow, amorphous residue was purified by column chromatography (hexane:EtOAc = 98:2) to afford compound **3** as a white solid.

Hexakis (2,3-di-O-propyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (4)

Compound **1** (0.3mmol, 0.5g) was dissolved in 20mL of anhydrous DMF and cooled to 0°C. NaH (60% in mineral oil, 12.7mmol, 0.30g, 42eq) was washed with hexane and added to the reaction mixture, which was stirred for 1 hour. PrI (10.9mmol, 1.1mL, 36eq) was added dropwise and then the reaction was left overnight. The same amount of NaH and PrI was added after every 24 hours for 4 days. Then MeOH was added dropwise, the mixture was evaporated and the residue was dissolved in DCM and washed successively with water, aqueous $Na_2S_2O_3$ and water again. The yellow, amorphous residue was purified with column chromatography (hexane:EtOAc=9:1) and the white crystalline compound **4** was separated.

Hexakis (2,3-di-O-butyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (5)

Compound **1** (0.9mmol, 1.5g) was dissolved in 60mL of anhydrous DMF and cooled to 0°C. NaH (60% in mineral oil, 38mmol, 0.91g, 42eq) was added to the solution and stirred for 1h. (38mmol, 4.1mL, 42eq) of BuBr was added to the reaction mixture dropwise, with 2 g KI. Then the reaction was left under reflux overnight. Reaction was followed by TLC (hexane:EtOAc=98:2), and if needed, the same amount of reactants was added in the same order. MeOH was added dropwise, and the mixture was concentrated. The residue was dissolved in $CHCl_3$ and washed successively with water, $Na_2S_2O_3$ and water again. The organic layer was dried on Na_2SO_4 and evaporated. The residue was the subject for column chromatography (hexane:EtOAc=6:4).

Hexakis (2,3-di-O-benzyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (6)

Compound **1** (1.2mmol, 2.0g) was dissolved in 60mL of anhydrous DMF. NaH (0.05mol, 1.2g, 42eq, 60% in mineral oil) was added to the solution at 0°C. After 1 hour stirring, BnBr (5.4mmol, 5.9mL, 42eq) was added dropwise and the reaction was left overnight. The same amount of reactants was added as previously in every 24 hours, until no change was seen in the distribution of the main and by-products. MeOH was added, then the solution was poured into cold water. The precipitate was filtered and washed with cold water. It was dissolved in $CHCl_3$, washed with water, dried on Na_2SO_4, and evaporated. The residue was subjected to column chromatography ($CHCl_3$:MeOH=8:0.5) to afford compound **6**.

Hexakis (2,3-di-O-methyl)-α-cyclodextrin (7)

Compound **2** (0.38mmol, 0.79g) was dissolved in 20mL of distilled THF. TBAF (4.3mmol, 4.3mL, 12eq, 1M in THF) was added dropwise, and the reaction was stirred under N_2 overnight. The reaction mixture was concentrated then purified by column chromatography (EtOAc:MeOH = 6:4), resulting to a white solid compound **7.**

Hexakis (2,3-di-O-ethyl)-α-cyclodextrin (8)

Compound **3** (0.15mmol, 0.3g) was dissolved in 15mL of distilled THF under N_2. TBAF (1.8mmol, 1.8mL, 12eq, 1M in THF) was added dropwise, and the reaction was stirred overnight under reflux. The mixture was concentrated, dissolved in DCM, washed successively with water. The organic layer was dried on Na_2SO_4 and evaporated. The yellowish residue was purified by flash chromatography (CombiFlash, DCM:MeOH =9:1), leading to compound **8**, as a white solid.

Hexakis (2,3-di-O-propyl)-α-cyclodextrin (9)

Compound **4** (0.33mmol, 0.71g) was dissolved in 20mL of distilled THF, and (3.95mmol, 3.95mL, 12eq, 1M in THF) TBAF was added dropwise. The reaction was stirred under reflux and N_2 gas overnight, then the reaction mixture was concentrated. The residue was dissolved in $CHCl_3$ and washed with water, successively. The organic layer was dried on Na_2SO_4 and evaporated. Then column chromatography was performed (DCM:MeOH = 9:1) and compound **9** was successfully separated.

Hexakis (2,3-di-O-butyl)-α-cyclodextrin (10)

Compound **5** (0.27mmol, 0.63g) was dissolved in 50mL of distilled THF, and TBAF (3.24mmol, 3.24mL, 12eq, 1M in THF) was added dropwise under N_2 gas, and the reaction was stirred under reflux overnight. The reaction mixture was concentrated, the residue was dissolved in $CHCl_3$ and washed with water. The organic layer was dried on Na_2SO_4 and concentrated. The residue was purified by flash chromatography (CombiFlash, $CHCl_3$:MeOH = 97:3), and compound **10** was separated.

Hexakis (2,3-di-O-benzyl)-α-cyclodextrin (11)

Compound **6** (0.62mmol, 1.69g) was dissolved in 30mL of distilled THF. TBAF (7.4mmol, 7.4mL, 12eq, 1M in THF) was added dropwise and the reaction was kept under reflux and N_2 gas overnight. The solution was concentrated, the residue was dissolved in $CHCl_3$, washed successively with water, dried on Na_2SO_4 and evaporated. The residue was purified with column chromatography ($CHCl_3$:MeOH = 9:0.5) leading to compound **11**.

Hexakis (2,3-di-O-methyl-6-O-sulfate)-α-cyclodextrin, DS=6 (12)

20mL of anhydrous pyridine was cooled to -10°C and 1mL of HSO_3Cl (15.6mmol, 60eq) was added dropwise, while the temperature was kept below 10°C. Then the mixture was heated to 70°C, and compound **7** (0.26mmol, 0.3g) was added. The reaction was stirred at 70°C overnight. The reaction mixture was neutralized with $NaHCO_3$ and evaporated. The residue was dissolved in water and filtrated through celite. The filtrate was dialyzed (MWCO=1kDa) and lyophilized to obtain the white, solid compound **12**.

Hexakis (2,3-di-O-ethyl-6-O-sulfate)-α-cyclodextrin, DS=5.46 (13)

0.35mL (5.32mmol, 70eq) of HSO_3Cl was added dropwise to 8ml of anhydrous pyridine cooled to -10°C, and the temperature was kept under 10°C. The mixture was heated to 70°C, and compound **8** (0.076mmol, 0.10g), dissolved in 2mL of pyridine, was added to the mixture. The reaction was stirred at 70°C under N_2 gas overnight. The reaction mixture was cooled on ice, 3mL of water was added, and was neutralized with NaOH solution, then concentrated. The formed solid was dissolved in water and dialyzed (MWCO=1kDa), then filtrated through celite. The filtrate was concentrated and projected to C18 flash column chromatography (H_2O and MeOH) and freeze drying, to obtain the white, solid compound **13**.

Hexakis (2,3-di-O-propyl-6-O-sulfate)-α-cyclodextrin, DS=5.14 (14)

0.3mL of HSO_3Cl (4.69mmol, 70eq) was added dropwise to 8mL of anhydrous pyridine cooled to -10°C, and the temperature was kept under 10°C. The mixture was heated to 70°C, and compound **9** (0.067mmol, 0.10g), dissolved in 2mL of pyridine, was added to the mixture. The reaction was stirred at 70°C under N_2 gas overnight. The reaction mixture was cooled on ice, 3mL of water was added, and neutralized with NaOH solution, then concentrated. The residue was dialyzed (MWCO=1kDa), and freeze dried. The solid was dissolved in water and it gave an opalescent solution, but the addition of base resulted a transparent solution. It was filtered through celite to remove the remaining pyridine salts. The filtrate was dialyzed again and freeze dried to obtain the white solid compound **14**.

Hexakis (2,3-di-O-butyl-6-O-sulfate)-α-cyclodextrin, DS=5.70 (15)

0.3mL of HSO_3Cl (4.2mmol, 70eq) was added dropwise to 10mL of anhydrous pyridine cooled to -10°C, and the temperature was kept under 10°C. Then the mixture was heated to 70°C and compound **10** (0.06mmol, 0.10g) dissolved in 2mL of pyridine was added dropwise. The reaction was stirred at 70 °C under N_2 gas overnight. The reaction mixture was cooled on ice and 3mL of water was added and neutralized with NaOH solution, then concentrated. The formed solid was dissolved in MeOH and filtrated through hydrophobic membrane (1µm pore diameter). 2M NaOH solution was added dropwise to the filtrate until white precipitate appeared. The precipitate was filtrated through hydrophobic membrane (0.2µm pore diameter). The filtrate was dried and the same procedure was repeated 2 more times. The precipitate was dissolved in water and dialyzed (MWCO=1kDa), later freeze dried. It gave a white solid product of **15**.

Hexakis (2,3-di-O-methyl-6-O-propanesultone)-α-cyclodextrin, DS= 2.91 (16)

Compund **7** (1.51mmol, 1.72g) was dissolved in 200mL of distilled THF under N_2 gas. NaH (60% in mineral oil, 75.5mmol, 1.8g, 50eq) was washed in hexane and added to the solution, then stirred for 15min at room temperature. 18-crow-6 (37.75mmol, 9.98g, 25eq) was added slowly to the reaction mixture. 30min later 1,3-propanesultone (52.85mmol, 6.5g, 35eq) was added for 30 minutes. The reaction was left overnight at room temperature, under N_2 gas. MeOH was added to decompose the non-reacted NaH, and then it was evaporated. The white solid residue was extracted with DCM to remove the crown ether, filtrated through resin, then dialyzed (MWCO=1kDa) and lyophilized.

Hexakis (2,3-di-O-ethyl-6-O-propanesultone)-α-cyclodextrin, DS=4.64 (17)

Compound **8** (0.23mmol, 0.3g) was dissolved in 80mL of anhydrous THF under N_2 gas. NaH (60% in mineral oil 11.46mmol, 0.27g, 50eq) was washed in hexane and added to the solution, then stirred for 15min at room temperature. 18-crow-6 (5.73mmol, 1.51g 25eq) was added slowly to the reaction mixture. 30min later 1,3-propanesultone (6.88mmol, 0.84g, 30eq) was added, for 30 minutes. The reaction was left overnight at room temperature, under N_2 gas. MeOH was added, then the mixture was concentrated. The white solid residue was extracted with DCM to remove the crown ether and then was dialyzed (MWCO=1kDa) and lyophilized. The residue was further purified by reversed phase C-18 flash column chromatography to separate compound **17**.

Hexakis (2,3-di-O-propyl-6-O-propanesultone)-α-cyclodextrin, DS=4.19 (18)

Compound **9** (0.32mmol, 0.4g) was dissolved in 70mL of anhydrous THF under N_2 gas. NaH (60% in mineral oil, 16.35mmol, 0.39g, 50eq) was washed in hexane and added to the solution, then stirred for 15min at room temperature. 18-crow-6 ether (8.18mmol, 2.16g, 25eq) was added slowly to the reaction mixture. 30min later 1,3-propanesultone (9.81mmol, 1.20g, 30eq) was added for 30 minutes. The reaction was left overnight at room temperature, under N_2 gas. MeOH was added, and then the mixture was concentrated. The white solid residue was dialyzed (MWCO=1kDa), filtrated through resin and then it was lyophilized to obtain compound **18**.

Hexakis (2,3-di-O-butyl-6-O-propanesultone)-α-cyclodextrin, DS= 3.84 (19)

Compound **10** (0.06mmol, 0.1g) was dissolved in 20mL of anhydrous THF under N_2 gas. NaH (60% in mineral oil 3mmol, 0.07g, 50eq) was washed in hexane, added to the solution and stirred for 15min at room temperature. Then 18-crow-6 (1.5mmol, 0.4g, 25eq) was added slowly to the reaction mixture. 30min later 1,3-propanesultone (2.1mmol, 0.26g, 35eq) was added for 30 minutes. The reaction was left overnight at room temperature, under N_2 gas. MeOH was added, then the mixture it was concentrated. White solid formed. The crude product was dialyzed, and projected to reversed phase column chromatography.

Hexakis (2,3-di-O-benzyl-6-O-ethyl)-α-cyclodextrin (20)

Compound **11** (0.15mmol, 0.3g) was dissolved in 10mL of anhydrous DMF. NaH (60% in mineral oil, 2.63mmol, 0.06g, 18eq) was added at 0°C under N_2 gas. After 1h of stirring EtBr (5.26mmol, 0.4mL, 36eq) was added dropwise. The reaction was stirred under N_2 gas overnight. The reaction was stopped with MeOH, then the mixture was concentrated, the

residue was dissolved in $CHCl_3$ and washed successively with water, and saturated $Na_2S_2O_3$. The organic layer was dried on Na_2SO_4 and evaporated. Then, the residue was purified by column chromatography (DCM:MeOH=98:2) to obtain compound **20**.

Hexakis (2,3-di-O-benzyl-6-O-propyl)-α-cyclodextrin (21)

Compound **11** (0.20mmol, 0.4g) was dissolved in 40mL of anhydrous DMF. NaH (60% in mineral oil, 4.67mmol, 0.11g, 24eq) was added at 0°C. After 1h stirring PrI (8.17mmol, 0.80mL, 42eq) was added dropwise. The reaction was stirred under N_2 gas for 48 hours. MeOH was added, then the mixture was concentrated, the residue was dissolved in $CHCl_3$ and washed successively with water, and saturated $Na_2S_2O_3$. The organic layer was dried on Na_2SO_4 and evaporated. Then, the residue was purified with column chromatography (DCM:MeOH=98:2) to separate compound **21**.

Hexakis (2,3-di-O-benzyl-6-O-butyl)-α-cyclodextrin (22)

Compound **11** (0.15mmol, 0.3g) was dissolved in 20mL of anhydrous DMF. NaH (60% in mineral oil, 2.63mmol, 0.06g, 18eq) was added at 0°C. After 1h stirring BuBr (5.26mmol, 0.57mL, 36eq) was added dropwise. The reaction was stirred under N_2 gas for 4 days. The reaction was stopped with MeOH, then the mixture was concentrated, the residue was dissolved in $CHCl_3$ and washed successively with water, and saturated $Na_2S_2O_3$. The organic layer was dried on Na_2SO_4 and evaporated. Then, the residue was purified by column chromatography (DCM:MeOH=99:0.5) to separate compound **22**.

Hexakis (6-O-ethyl)-α-cyclodextrin (23)

Compound **20** (0.19mmol, 0.42g) was dissolved in 7mL of acetic acid and hydrogenated in the presence of 10% Pd/C (0.5g). The reaction mixture was filtrated through celite, washed with MeOH and concentrated. The residue was purified with column chromatography ($CHCl_3$:MeOH = 8:2) to obtain compound **23**.

Hexakis (6-O-propyl)-α-cyclodextrin (24)

Compound **21** (0.19mmol, 0.44g) was dissolved in 10mL of acetic acid and hydrogenated in the presence of 10% Pd/C (0.6g). The reaction mixture was filtered through celite and concentrated.

Hexakis (2,3,6-tri-O-ethyl)-α-cyclodextrin (25)

Dry native α-CD (0.51mmol, 0.5g) was dissolved in 80mL of 4/1 THF:DMSO mixture. NaH (60% in mineral oil, 12.85mmol, 0.31g, 25eq) was added at 0°C after removing the oil with hexane. After 1h stirring EtBr (12.85mmol, 0.96mL, 25eq) was added to the solution dropwise, at room temperature. The reaction was followed with TLC ($CHCl_3$:MeOH=8:2) and the same amount of EtBr was added until no change could be seen, meanwhile the temperature was kept at 45°C. The reaction was stopped by adding MeOH, then the reaction mixture was poured into ice-water, and washed with $CHCl_3$. The organic layer was washed with aqueous NH_4Cl, $Na_2S_2O_3$ and brine, then dried and concentrated. The residue was purified with column chromatography ($CHCl_3$:MeOH = 99:1).

Hexakis (2,3,6-tri-O-propyl)-α-cyclodextrin (26)

Dry native α-CD (0.72mmol, 0.7g) was dissolved in in 100mL of 4/1 THF:DMSO mixture. NaH (60% in mineral oil, 17.99mmol, 0.43g, 25eq) was added at 0°C after removing the oil with hexane. After 1h stirring PrI (17.99mmol, 1.75mL, 25eq) was added dropwise. The reaction was followed by TLC ($CHCl_3$:MeOH = 8:2) and the same amount of reactants was added until no change could be seen. Then the reaction was stopped by adding MeOH, then the reaction mixture was poured into ice-water, and washed with $CHCl_3$. The organic layer was washed with aqueous NH_4Cl, $Na_2S_2O_3$ and brine, then dried and concentrated. The residue was purified with column chromatography (cyclohexane:diethyl ether=7:3).

Hexakis (2,3,6-tri-O-butyl)-α-cyclodextrin (27)

Dry native α-CD (0.72mmol, 0.7g) was dissolved in 100mL of 4/1 THF:DMSO mixture. NaH (60% in mineral oil, 17.99mmol, 0.43g, 25eq) was added at 0°C after removing the oil with hexane. After 1h stirring BuBr (17.99mmol, 1.9mL, 25eq) was added to the solution dropwise. The reaction was followed by TLC ($CHCl_3$:MeOH = 8:2) and the same amount of reactants was added until no change could be seen. The reaction was stopped by adding MeOH, then the reaction mixture was poured into ice-water, and washed with $CHCl_3$. The organic layer was washed with aqueous NH_4Cl, $Na_2S_2O_3$ and brine, then dried and concentrated. After residue was purified with column chromatography (cyclohexane:diethyl ether=7:3)

XI. Results

A. α-cyclodextrins

1. Hemolytic activity

Native α-CD showed relatively high hemolytic effect as its HC_{50} value was 16 mM. Only two derivatives proved to have more harmful effect on RBCs, namely the TRIMEA which caused hemolysis at 1.9mM, followed by the succinyl derivative with an HC_{50} value of 9.6mM. (Figure 22 and Table 7) RAMEA caused hemolysis at similar concentration (15.5mM) to the parent molecule. The order of hemolytic effect in case of methylation compared to the native CD is: TRIMEA >> RAMEA ≥ native α-CD. Five derivatives out of the ten however, had no severe haemolytic activity up to 100mM, as their HC_{50} values could have not been defined under these circumstances. Among the non-ionic derivatives, HPACD and AcACD had no effect, and in case of the ionic derivatives, the HC_{50} value could be determined only for CMACDEp (24mM). [273]

Table 7 *Comparison of those α-CD derivatives' cytotoxicity which are commercially available, by different methods (MTT, RT-CES, hemolysis). Values are in mM, and expressed as mean ± SD.*

α-cyclodextrin derivative	IC_{50} (MTT)	IC_{50} (RT-CES)	HC_{50}
native	46.1±9.2	> 25	16.0±0.02
RAMEA	78.6±15.8	> 25	15.5±0.01
TRIMEA	1.8±0.8	> 1	1.9±0.01
HPACD	> 100	> 100	> 100
sulfated	> 100	>10	> 100
phosphated	7.8±8.6	>10	> 100
CMACD	> 100	> 25	> 100
SuACD	19.0±8.8	> 1	9.6±0.03
AcACD	> 100	> 100	> 100
CMACDEp	58.4±0.4	>10	24.5±0.01

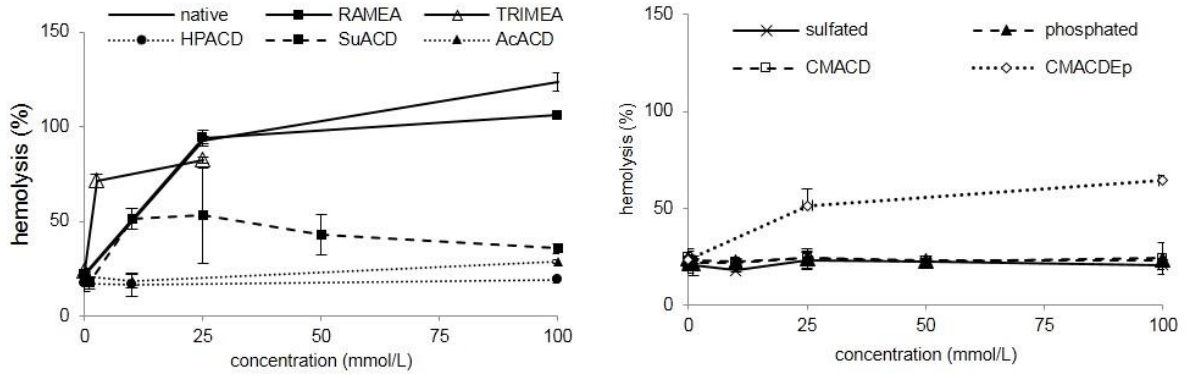


Figure 22 *Hemolytic effect of non-ionic (left) and ionic (right) α-CD derivatives on human RBCs. Hemolysis was expressed as the percentage of untreated control in the function of α-CD derivative concentration. Negative control: PBS. Positive control: purified water.*

2. MTT assay

MTT assays resulted in a similar toxic profile as evolved out of hemolysis tests, although some remarkable differences were observed. (Table 7) For example, the phosphated CD did not show haemolytic activity, but it was undoubtedly harmful on Caco-2 cells, with an IC_{50} value at 7.8mM. Regarding the methylated derivatives, the same toxic order was recognised as previously (TRIMEA>>RAMEA>>native), however this time RAMEA proved to be significantly less toxic (IC_{50}=78mM) than the parent α-CD (IC_{50}=46mM). (Figure 23) Among the non-ionic CDs, HPACD and AcACD did not have significant effect on the cell viability up to 100mM, similarly to the ionic CMACD and sulfated derivatives. CMACDEp with its IC_{50} at 58mM is classified as a mid-range element, possessing reduced toxicity compared to the parent molecule. [273]

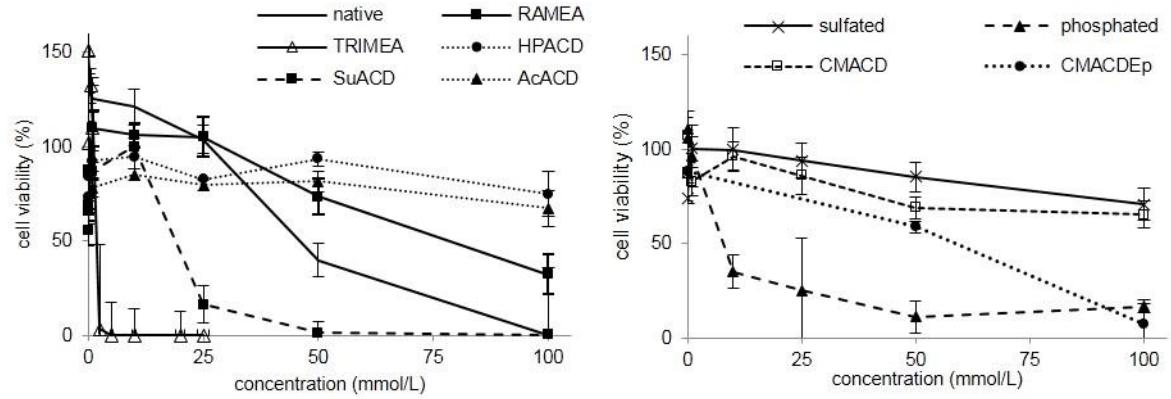


Figure 23 *Effect of non-ionic (left) and ionic (right) α-CD derivatives on Caco-2 cell viability, determined by MTT-test. Cell viability was expressed as the percentage of untreated control in the function of α-CD derivatives concentration. PBS served as negative control and Triton-X 100 (1% w/v) as positive control. Values presented are means ± SD.*

When only methylated derivatives were compared, the IC_{50} concentrations have increased as the number of methyl groups per glucose residue decreased. (Figure 24) The toxic effect decreased in the following order: TRIMEA (1.8mM) < DIMEA (36.4mM) < RAMEA (78.6mM) < PARMEA (87.1mM). The number of methyl groups per CD were 18, 12, ~11, ~9, respectively. The simultaneous presence of 6 sulfate group at the C-6 position resulted in severe increase of toxic effect, namely, the IC_{50} value of **12** was determined at the low 9.26mM concentration. However, **13** which differs in one methylene group at both C-2 and C-3 position from **12,** showed milder toxic effect with its IC_{50} value at 34.0mM.

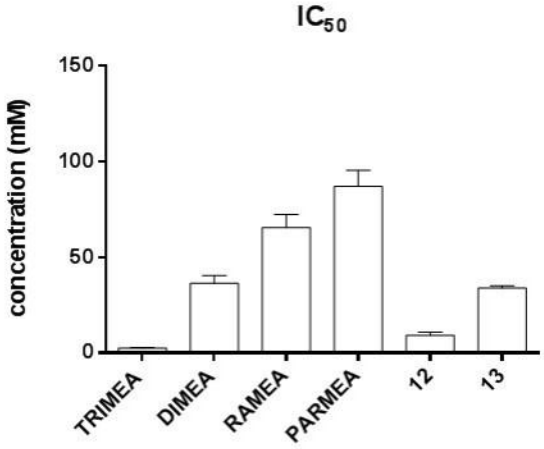


Figure 24 *Comparison of cytotoxicity of methylated α-CDs which differ in the degree of substitution. TRIMEA has 18, DIMEA 12, RAMEA ~11 and PARMEA ~9 methyl groups.* ***12*** *differs from DIMEA in 6 sulfate group at the C-6 position, and* ***13*** *differs from* ***12*** *in the ethyl groups at C-2 and C-3 positions. Values presented are means ± SD. PBS served as negative control and Triton-X 100 (1% w/v) as positive control. All data were obtained from two to three independent biological replicates and in the same experiments two to three parallel concentrations were measured.*

The toxic effect of RAMEA and its pre-formed inclusion complex with phosphatidylcholine was compared by MTT assay on Caco-2 cells. (Figure 25) RAMEA had its IC_{50} value at 78.6mM, and the complexation resulted in the decrease of cytotoxicity. When phosphatidylcholine was complexed within the CD cavity, there was no definable IC_{50} up to 100mM, under the same circumstances. [273] This difference verifies indirectly the theory of the phospholipid extraction from the cell membrane by α-CDs, which gives rise to the disruption of membrane integrity, thus cell lysis.

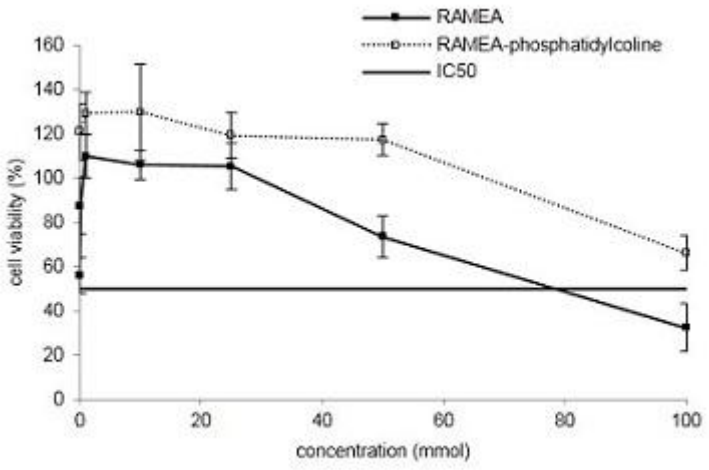
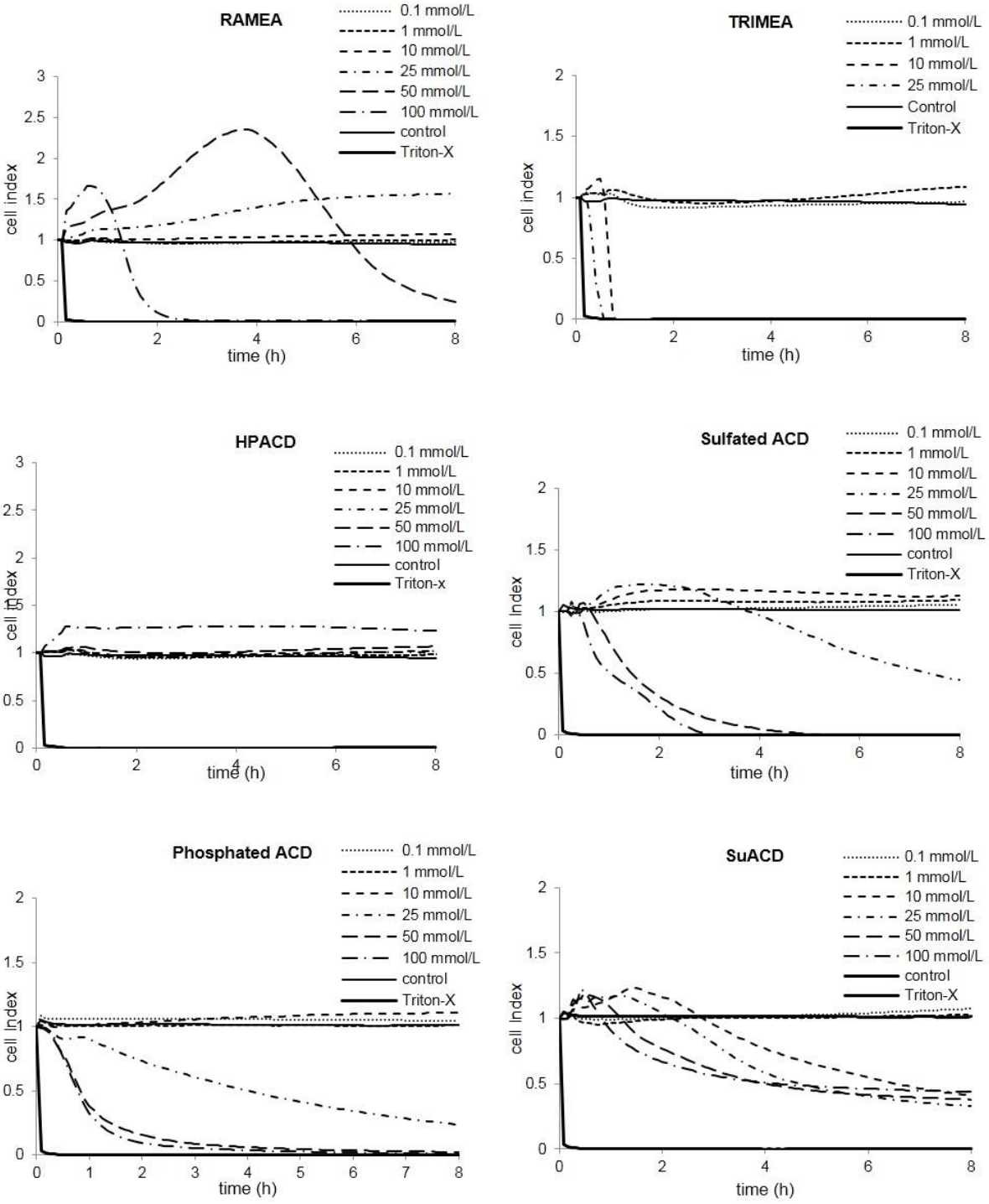


Figure 25 *Comparison of toxicity of RAMEA and RAMEA–phosphatidylcholine complex on Caco-2 cells. Cell viability was expressed as the percentage of untreated control in the function of RAMEA concentration. Values presented are mean ± SD. All data were obtained from three independent biological replicates and in the same experiment three parallel concentrations were measured.*

3. RT-CES

In case of RT-CES, eight derivatives by the ten showed measurable IC_{50} value. (Table 7, Figure 26) Only two derivatives remained to be non-toxic up to 100mM, namely the HPACD and AcACD, which were proved to be safe also by MTT assay. The methylated derivatives followed the conventional order of TRIMEA>RAMEA, thus TRIMEA possessed more severe toxicity (IC_{50}>1mM) compared to RAMEA or the native α-CD, while RAMEA had similar effect on Caco-2 cells as the parent molecule. (IC_{50}>25mM). SuACD as a non-ionic representative had significant toxic effect over 1mM. Among the ionic derivatives CMACD and sulphated ACD had no defined IC_{50} value up to 100mM by MTT assay, although they significantly reduced the viable cell number this time. CMACD proved to be safe up to 25mM, and sulfated ACD only up to 10mM. Phosphated ACD showed similar effects with both

methods (IC_{50}>10mM). CMACDEp caused cell death of more than 50% of the cell population over 10mM. [273]



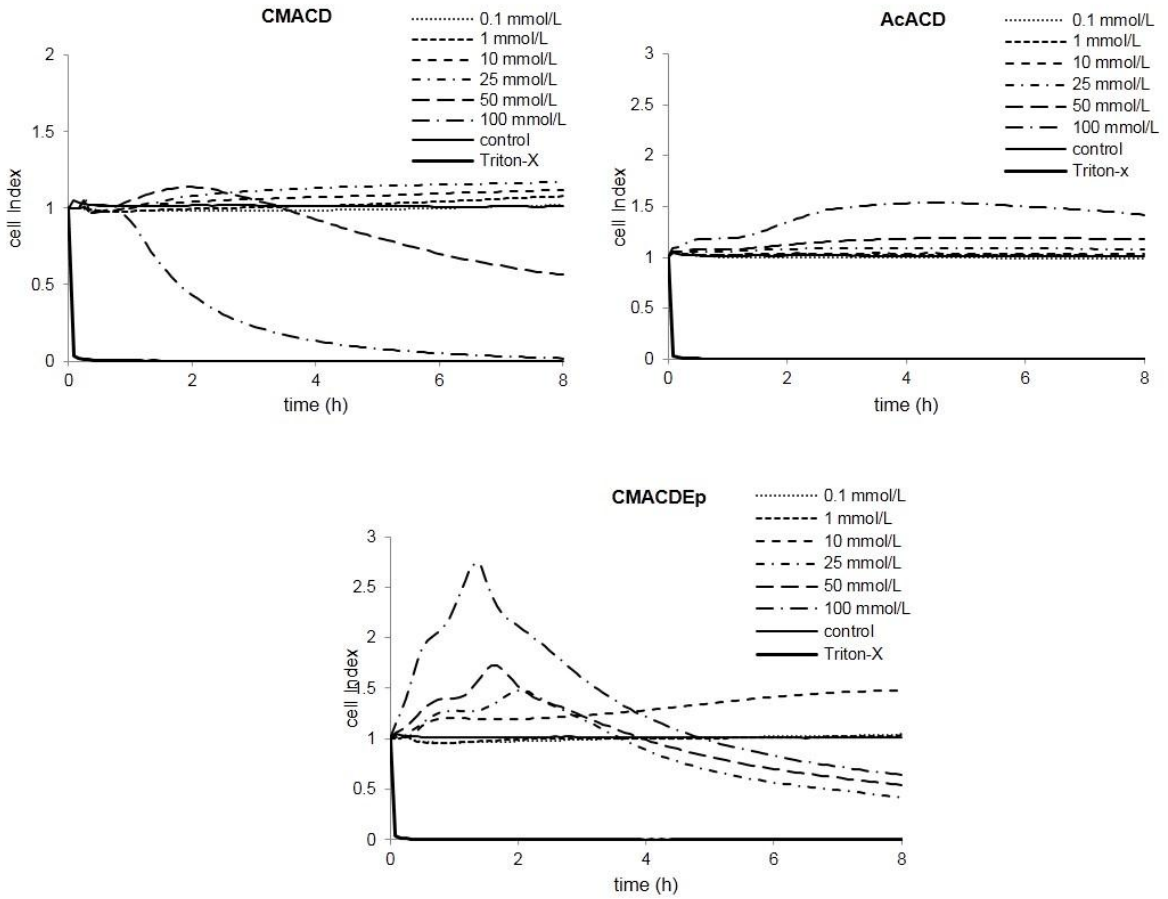


Figure 26 *Effect of α-CD derivatives on Caco-2 cell viability, determined by Real Time Cell Electronic Sensing. Changes in cell index indicating viability of Caco-2 cells up to 8h treatment with different α-CD derivatives. Data are presented as mean ± S.D. n=3 parallel samples. PBS served as negative control and Triton-X 100 (1% w/v) as positive control.*

4. Synthesis

Synthetic approaches to per-2,3-alkylated and per-6-alkylated α-CDs started with the protection of the primary hydroxyls with TBDMS group. This reaction gave a high yield (72%) and provided the starting material to produce compounds **2-24.**

The yield of secondary face alkylations' varied between 27 and 82%. The reaction time and reactant equivalents had to be increased with the length of the alkyl chain. Each crude product contained several under-substituted components, thus flash chromatography was needed to separate the main compounds, which is further lowered the yield. The comparison of the ^{1}H and ^{13}C NMR spectra of compound **1** and **2** shows the peaks corresponding to the methyl groups at C-2 and C-3 positions. (Figures 27 and 28)

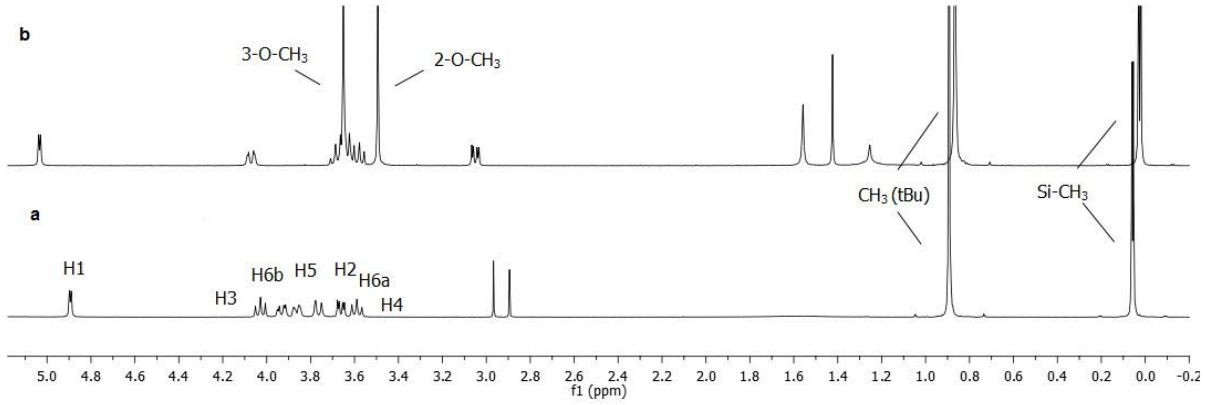


***Figure 27** ^{1}H NMR (400Hz) spectra of: a) compound **1**, b) compound **2**.*

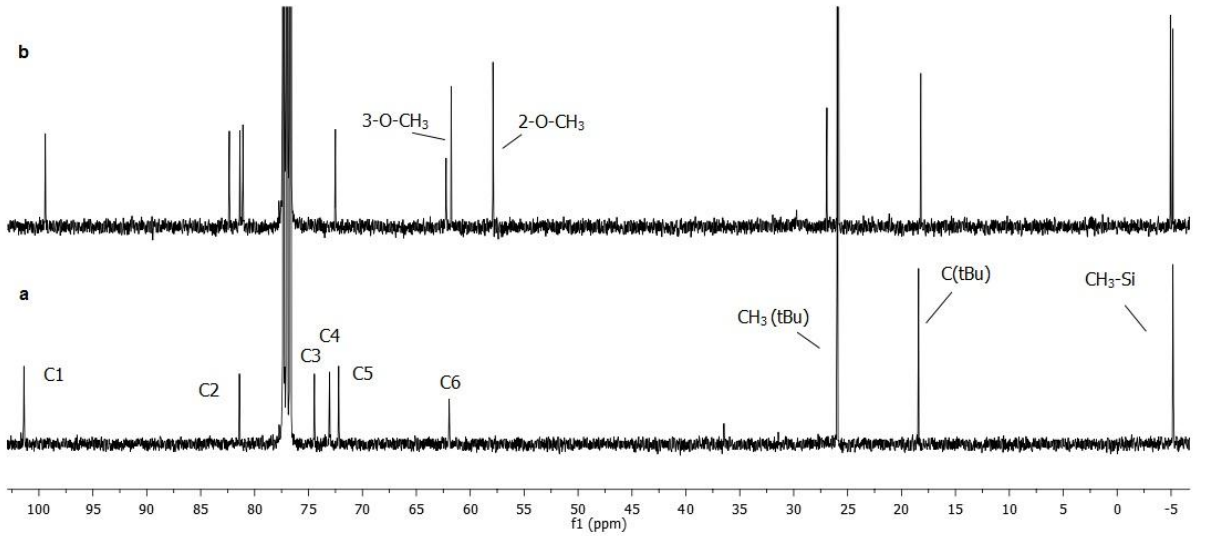


***Figure 28** ^{13}C NMR (100Hz) spectra of: a) compound **1**, b) compound **2**.*

The final step of secondary face substitution (compounds **7-11**) was the removal of the silyl ethers from the C-6 positions. It was achieved with TBAF, giving a final yield varying between 47 and 79%. Product were subjected to column chromatography for final separation and purification. The increasing number of methylene groups on the side chains can be seen on the ^{1}H and ^{13}C NMR spectra in Figures 29 and 30, respectively.

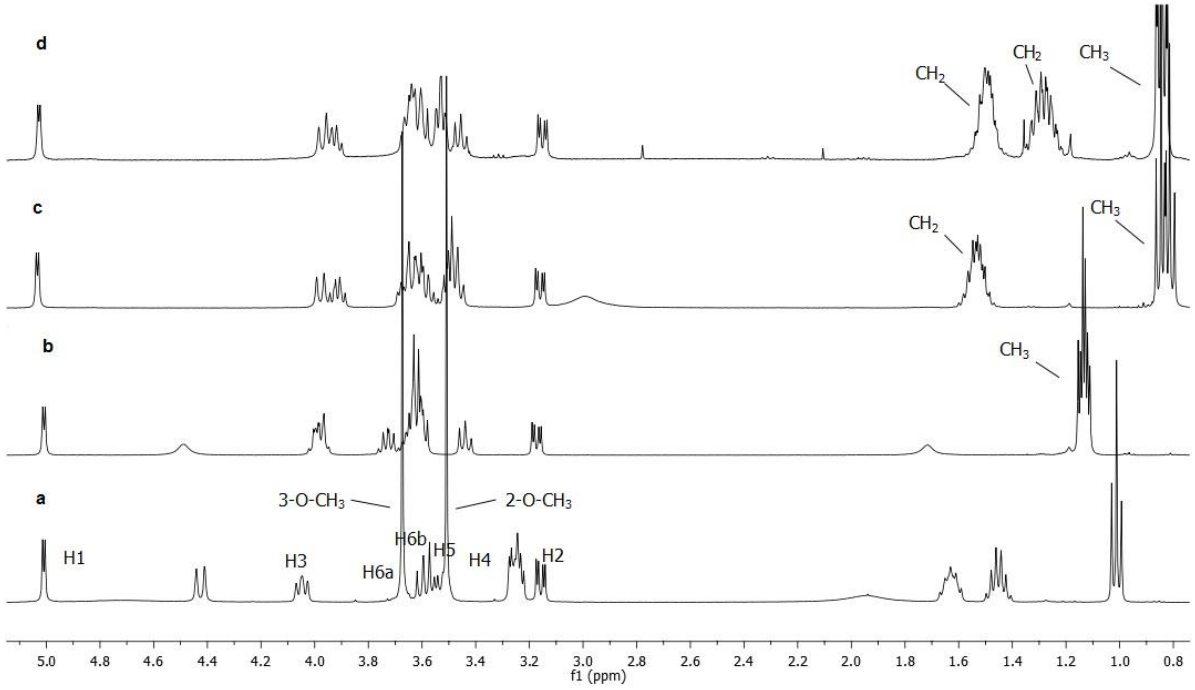


Figure 29 *^{1}H NMR (400Hz) spectra of: a) compound **7**, b) compound **8**, c) compound **9**, d) compound **10**.*

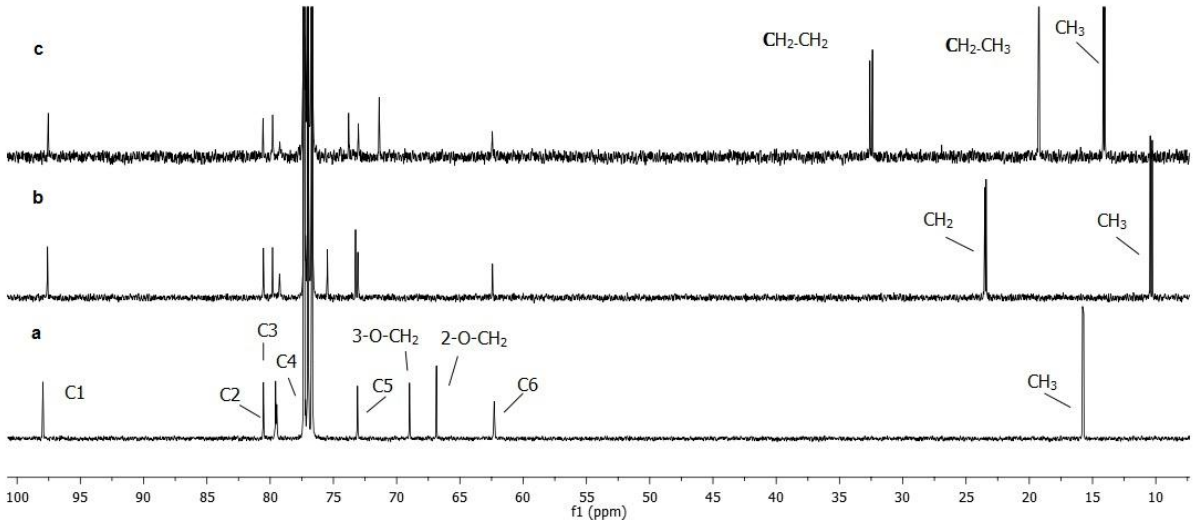


Figure 30 *^{13}C NMR (100Hz) spectra of: a) compound **8**, b) compound **9**, c) compound **10**.*

Due to the lack of solubility of 2,3-di-O-alkylated derivatives, they were further modified with sulfate and sulfopropyl substitution.

Sulfate substitution gave high DSs (6, 5.46, 5.14 and 5.70 referring to **12, 13, 14, 15**, respectively). The main difficulty was caused by the problematic removal of pyridine salts from the crude compound. The application of celite as filtrating agent gave the solution in case of **12** and **13**. Compound **14** was subjected to C18 column chromatography, and **15** was precipitated from its basic aqueous solution with MeOH. Figure 31 shows the ^{1}H NMR spectra of compound **12** compared to its starting material (compound **7**).

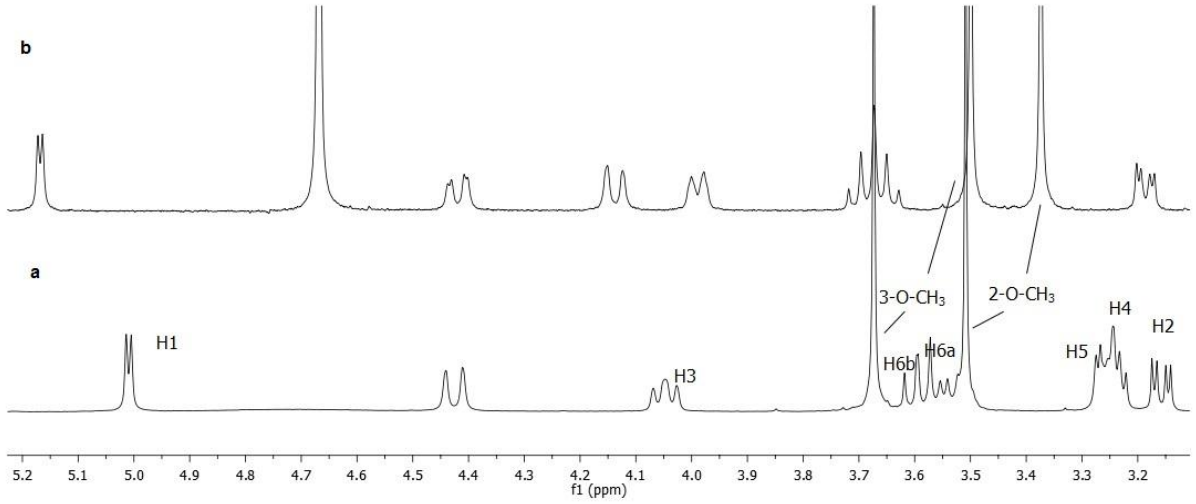


Figure 31 *^{1}H NMR (400Hz) spectra of: a) compound **7** in $CDCl_3$ and b) compound **12** in D_2O.*

Sulfopropylation resulted products with the following DSs: 2.91, 4.64, 4.19 and 3.84 (referring to compound **16**, **17**, **18** and **19**). (Figure 32) The two main obstacles which hampered the production of pure compounds were the low DSs, thus the presence of mainly under-substituted products, and the difficulties in removing the sulfopropyl salts from the crude product. We suggest, that it is caused by the complexation of sulfopropyl salts within the CD cavity.

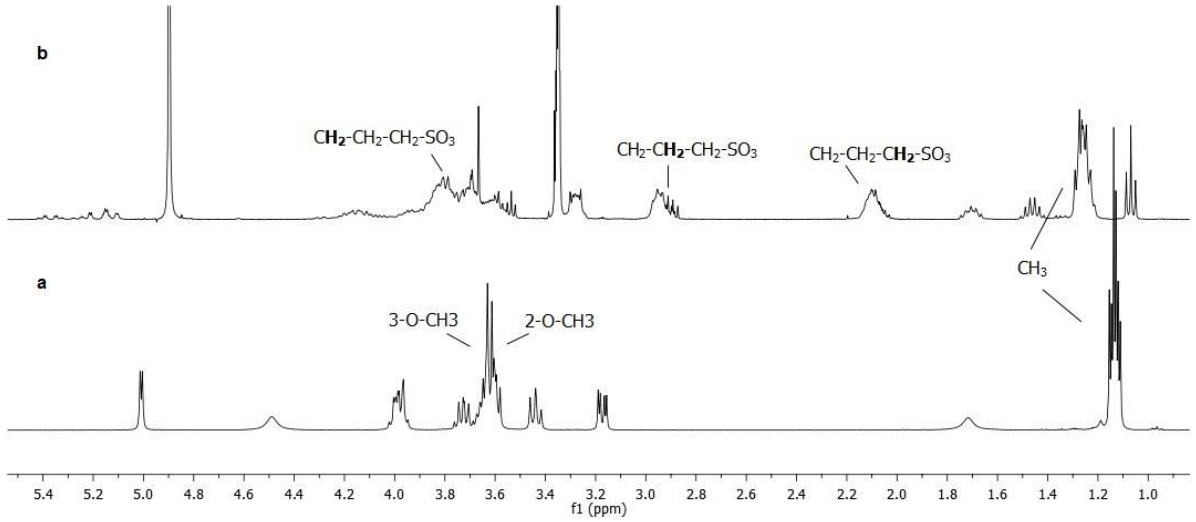
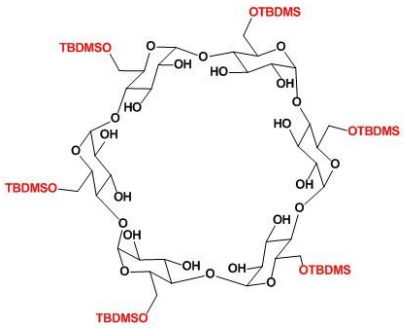


Figure 32 *^{1}H NMR (400Hz) spectra of: a) compound **8** in $CDCl_3$ and b) compound **17** in MeOD.*

In per-6-alkylation, the 2,3-di-O-butyl-6-O-alkyl intermediates (**20**, **21** and **22**) were produced with 85, 84 and 34% yield, respectively. The formation of compounds **23** and **24** did not result products in satisfactory amounts (e.g. in case of **23** the yield was 37%), thus it did allow us to perform further tests with them. Similar problems arose when complete per-substitution was approached (compounds **25-27**). The intense presence of by-products, containing 1-17 alkyl groups provided serious challenges in the purification step. When the final product was successfully separated, the yield was not satisfactory, and added to this, the lack of free hydroxyls resulted in low aqueous solubility.

5. Characterisation of synthetized derivatives

Hexakis (6-O-tert-butyldimethylsilyl)-α-cyclodextrin (1)



$C_{72}H_{144}O_{30}Si_6$
EM=1656.84

Yield = 72%

Rf=0.47 ($CHCl_3$:MeOH:water = 50:10:1)

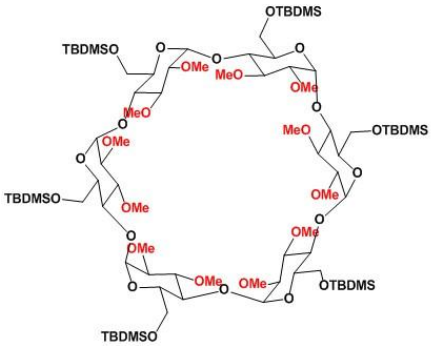
T_m= 315.9 ºC

Mass ESI (m/z)= [$M+H]^+$ 1658.8, **[**$M+H+Na]^{2+}$ 840.9; **[**$M+H+K]^{2+}$ 848.9

^{1}H NMR (CDCl$_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.06 (d, 36H, Si-CH_3, J=2.8Hz), 0.89 (s, 54H, CH_3, (tBu)), 3.57 (t, 6H, H-4, J=9Hz), 3.66 (dd, 6H, H-2, J=3.2Hz and 9.6Hz), 3.76 (d, 6H, H-6a, J=10Hz), 3.87 (d, 6H, H-5, J=10Hz), 3.93 (dd, 6H, H-6b, J=3.4Hz and 10Hz), 4.03 (t, 6H, H-3, J=9Hz), 4.89 (d, 6H, H-1, J=3.2Hz)

^{13}C NMR (CDCl$_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): -5.18 (CH_3-Si), -5.21 (CH_3-Si), 18.42 (C (tBu)), 25.97 (CH_3 (tBu)), 61.95 (C-6), 72.20 (C-5), 73.04 (C-4), 74.45 (C-3), 81.40 (C-2), 101.39 (C-1)

Hexakis (2,3-di-O-methyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (2)



$C_{84}H_{168}O_{30}Si_6$
EM=1825.02

Yield = 55%

Rf=0.69 (cyclohexane:EtOAc = 7:3)

T_m= 204.5 ºC

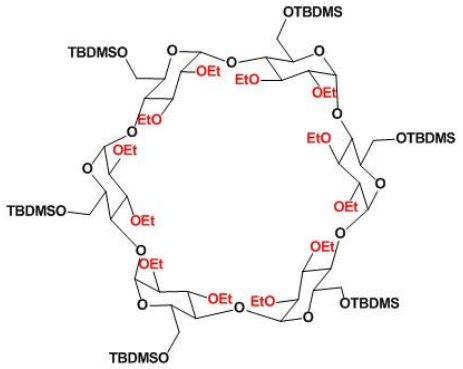
Mass ESI (m/z)= [$M+2Na]^{2+}$ 936, **[**$M+Na]^+$ 1849.0, **[**$M+H]^+$ 1827.0

^{1}H NMR (CDCl$_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.19 (d, 36H, Si-CH_3, J=2.8Hz), 0.87 (s, 54H, CH_3 (tBu)), 1.19-1.23 (m, 36H, 2-O-CH_2-C*H*$_3$, 3-O-CH_2-C*H*$_3$),

3.14 (dd, 6H, H-2, J=3.2Hz and 9.6 Hz), 3.54-3.75 (m, 30H, H-4, 2-O-C*H*$_2$-CH$_3$, 3-O-C*H*$_2$-CH$_3$), 3.79-3.91 (m, 12H, H-5, H-6a), 4.05-4.19 (m, 12H, H-3, H-6b), 5.16 (d, 6H, H-1, J=3.2Hz)

^{13}C NMR (CDCl$_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): -5.05 (CH$_3$-Si), -4.75 (CH$_3$-Si), 15.76 (2-O-CH$_2$-*C*H$_3$), 15.83 (3-O-CH$_2$-*C*H$_3$), 18.30 (C (tBu)), 25.95 (CH$_3$ (tBu)), 62.35 (C-6), 66.45 (2-O-CH$_2$), 68.94 (3-O-CH$_2$), 72.39 (C-5), 77.55 (C-4), 79.99 (C-3), 80.49 (C-2), 97.90 (C-1)

Hexakis (2,3-di-O-ethyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (3)



$C_{96}H_{192}O_{30}Si_6$
EM=1993.37

Yield = 81.9%

Rf=0.45 (hexane:EtOAc = 98:2)

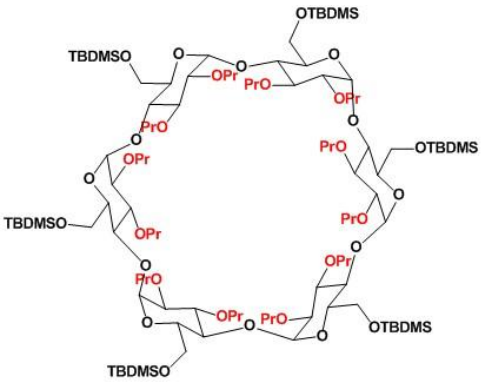
T$_m$= 200.9 ºC

Mass ESI (m/z)= [M+H]$^+$ 1995.2, [M+Na]$^+$ 2017.2

^{1}H NMR (CDCl$_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.19 (d, 36H, Si-CH$_3$, J=2.8Hz), 0.87 (s, 54H, CH$_3$ (tBu)), 1.19-1.23 (m, 36H, 2-O-CH$_2$-C*H*$_3$, 3-O-CH$_2$-C*H*$_3$), 3.14 (dd, 6H, H-2, J=3.2Hz and 9.6 Hz), 3.54-3.75 (m, 30H, H-4, 2-O-C*H*$_2$-CH$_3$, 3-O-C*H*$_2$-CH$_3$), 3.79-3.91 (m, 12H, H-5, H-6a), 4.05-4.19 (m, 12H, H-3, H-6b), 5.16 (d, 6H, H-1, J=3.2Hz)

^{13}C NMR (CDCl$_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): -5.05 (CH$_3$-Si), -4.75 (CH$_3$-Si), 15.76 (2-O-CH$_2$-*C*H$_3$), 15.83 (3-O-CH$_2$-*C*H$_3$), 18.30 (C (tBu)), 25.95 (CH$_3$ (tBu)), 62.35 (C-6), 66.45 (2-O-CH$_2$), 68.94 (3-O-CH$_2$), 72.39 (C-5), 77.55 (C-4), 79.99 (C-3), 80.49 (C-2), 97.90 (C-1)

Hexakis (2,3-di-O-propyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (4)



$C_{108}H_{216}O_{30}Si_6$
EM=2161.40

Yield = 27%

Rf=0.50 (hexane:EtOAc =9:1)

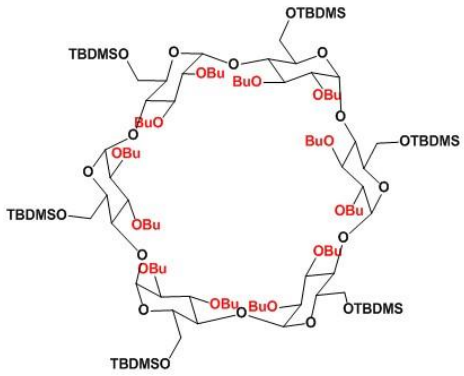
T_m= 209.7 ºC

Mass ESI (m/z)= [$M+H]^+$ 2163.4, **[**$M+Na]^+$ 2185.4

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.02 (d, 6H, Si-CH_3, J=2.4Hz), 0.86-0.93 (m, 90H, CH_3 (tBu), 2-O-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-C*H_3*), 1.58-1.66 (m, 4H, 2-O-CH_2-*CH_2*-CH_3, 3-O-CH_2-*CH_2*-CH_3), 3.12 (dd, 6H, H-2, J=3.2Hz and 9.6Hz), 3.50-3.76 (m, 36H, H-5, H-4, 2-O-C*H_2*-CH_2-CH_3, 3-O-C*H_2*-CH_2-CH_3), 3.91-4.05 (m, 6H, H-6a, H-6b), 4.20 (d, 6H, H-3, J=9.6Hz), 5.20 (d, 6H, H-1, J=3.2Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): -5.6 (CH_3-Si), -4.71 (CH_3-Si), 10.21 (2-CH_3 (Pr)), 10.47 (3-CH_3 (Pr)), 18.29 (C (tBu)), 23.47 (2-O-CH_2-*C*H_2-CH_3 (Pr)), 23.56 (3-O-CH_2-*C*H_2-CH_3), 25.96 (CH_3 (tBu)), 62.44 (C-6), 72.36 (C-5), 72.92 (2-O-CH_2), 75.44 (3-O-CH_2), 77.10 (C-4), 80.16 (C-3), 80.62 (C-2), 97.33 (C-1)

Hexakis (2,3-di-O-butyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (5)



$C_{120}H_{240}O_{30}Si_6$
EM=2329.59

Yield=50.1%

Rf=0.43 (hexane:EtOAc =98:2)

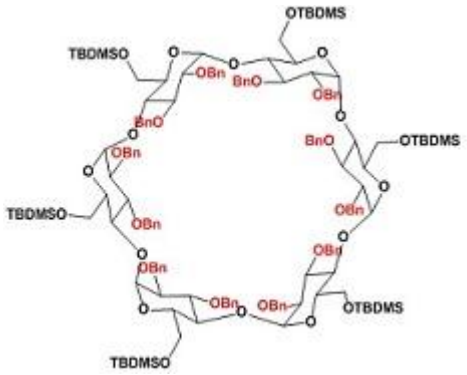
T_m= 211.4 ºC

Mass ESI (m/z)= [$[M+2H]^{++}$ 1166.8, **[**$[M+H]^{+}$ 2331.6, **[**$[M+Na]^{+}$ 2353.6

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.036 (d, 36H, Si-CH_3, J=2.8Hz), 0.89 (m, 90H, CH_3 (tBu), 2-O-CH_2-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-CH_2-C*H_3*), 1.26-1.43 (m, 24H, 2-O-CH_2-CH_2-C*H_2*-CH_3, 3-O-CH_2-CH_2-C*H_2*-CH_3), 1.52-1.63 (m, 24H, 2-O-CH_2-*CH_2*-CH_2-CH_3, 3-O-CH_2-*CH_2*-CH_2-CH_3), 3.12 (dd, 6H, H-2, J=3.6Hz and 10Hz), 3.55-3.65 (m, 24H, 2-O-C*H_2*-CH_2-CH_2-CH_3, 3-O-C*H_2*-CH_2-CH_2-CH_3), 3.70-3.76 (m, 12H, H-4, H-5), 3.91-4.07 (m, 12H, H-6a, H-6b), 4.20 (d, 6H, H-3, J=10 Hz), 5.20 (d, 6H, H-1, J=3.6Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): -5.05 (CH_3-Si), -4.69 (Si-CH_3), 14.05 (2-CH_3 (Bu)), 14.19 (3-CH_3 (Bu)), 18.30 (C (tBu)), 19.24 (2-O-CH_2-CH_2-*C*H_2-CH_3), 19.26 (3-O-CH_2-CH_2-*C*H_2-CH_3), 25.97 (CH_3 (tBu)), 32.45 (2-O-CH_2-*C*H_2-CH_2-CH_3), 32.69 (3-O-CH_2-*C*H_2-CH_2-CH_3), 62.45 (C-6), 71.08 (2-O-CH_2), 72.32 (C-5), 73.71 (3-O-CH_2), 77.13 (C-4), 80.19 (C-3), 80.69 (C-2), 97.31 (C-1)

Hexakis (2,3-di-O-benzyl-6-O-tert-butylmethylsilyl)-α-cyclodextrin (6)



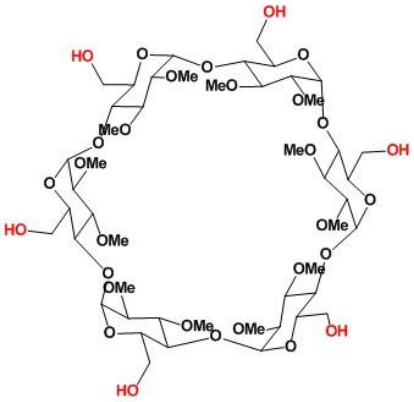
$C_{156}H_{216}O_{30}Si_6$
EM=2737.40

Yield = 33.0%

Rf=0.22 ($CHCl_3$:MeOH=8:0.5)

Mass ESI (m/z)= [$[M+H]^{+}$ 2740.3, **[**$[M+Na]^{+}$ 2762.3

Hexakis (2,3-di-O-methyl)-α-cyclodextrin (7) (DIMEA)



$C_{48}H_{84}O_{30}$
EM=1140.5

Yield=78%

Rf=0.41 (EtOAc:MeOH = 6:4)

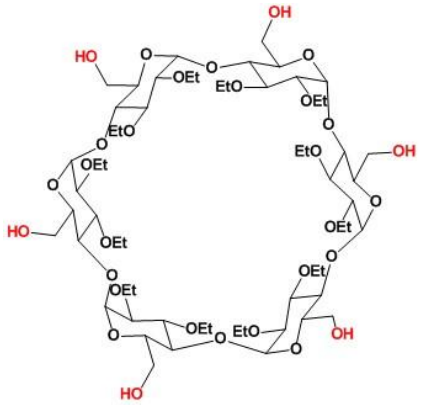
T_m= 199.65 ºC

Mass ESI (m/z)= [$[M+H]^+$ 1141.5, **[**$[M+Na]^+$ 1163.5

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 3.13 (dd, 6H, H-2, J=3.6Hz and 10Hz), 3.20-3.25 (m, 12H, H-4, H-5), 3.49 (s, 18H, 2-O-CH_3), 3.50-3.59 (m, 12H, H-3, H-6a), 3.65 (s, 18H, 2-O-CH_3), 4.023 (t, 6H, H-6b, J=8.4Hz), 4.99 (d, 6H, H-1, J=3.6Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): 57.95 (2-OCH_3), 61.72 (3-OCH_3), 62.98 (C-6), 73.56 (C-5), 81.64 (C-4), 82.23 (C-3), 83.66 (C-2), 99.27 (C-1)

Hexakis (2,3-di-O-ethyl)-α-cyclodextrin (8)



$C_{60}H_{108}O_{30}$
EM=1309.48

Yield=66.3%

Rf = 0.28 (DCM:MeOH = 9:1)

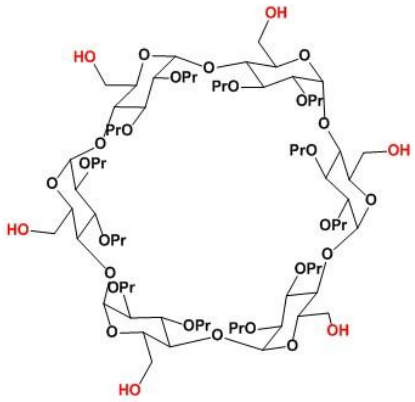
T_m= 159.3 ºC

Mass ESI (m/z)= [$[M-H]^-$ 1308.2, **[**$[M+Na]^+$ 1331.9

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 1.18-1.26 (m, 36H, 2-O-CH_2-C*H_3*, 3-O-CH_2-C*H_3*), 3.24 (dd, 6H, H-2, J=3.6Hz and 10Hz), 3.51 (t, 6H, H-4, J=8.8Hz), 3.65-3.74 (m, 30H, 2-O-C*H_2*, 3-O-C*H_2*, H-3), 3.75-3.83 (m, 6H, H-5), 4.01-4.09 (m, 12H, H-6a, H-6b), 5.07 (d, 6H, H-1, J=3.6Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): 15.70 (2-O-CH_2-*C*H_3), 15.79 (2-O-CH_2-*C*H_3), 62.29 (C-6), 66.86 (2-O-CH_2) 68.98 (3-O-CH_2), 73.09 (C-5), 79.48 (C-4), 79.57 (C-3), 80.53 (C-2), 97.96 (C-1)

Hexakis (2,3-di-O-propyl)-α-cyclodextrin (9)



$C_{72}H_{132}O_{30}$
EM=1476.88

Yield=46.75%

Rf=0.34 (DCM:MeOH = 9:1)

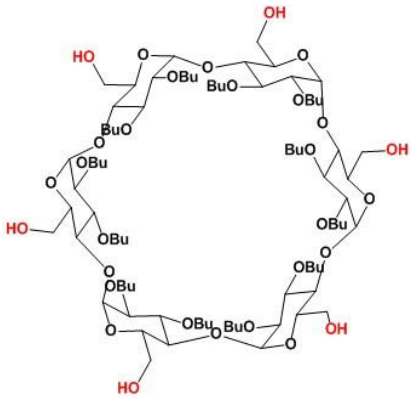
T_m= 227.5 ºC

Mass ESI (m/z)= [$[M-H]^-$ 1476.8, **[**$[M+Na]^+$ 1500.1

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.86-0.93 (m, 36H, 2-O-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-C*H_3*), 1.57-1.63 (m, 24H, 2-O-CH_2-C*H_2*-CH_3, 3-O-CH_2-C*H_2*-CH_3), 3.23 (dd, 6H, H-2, J=3.6Hz and 10Hz), 3.51-3.59 (m, 18H, H-4, H-3, H-5), 3.65-3.75 (m, 24H, 2-O-*CH_2*-CH_2-CH_3, 3-O-*CH_2*-CH_2-CH_3), 3.98-4.06 (m, 12H, H-6a, H-6b), 5.10 (d, 6H, H-1, J=3.6Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): 10.28 (2-CH_3 (Pr)), 10.44 (3-CH_3 (Pr)), 23.41 (2-CH_2 (Pr)), 23.53 (3-CH_2 (Pr)), 62.42 (C-6), 73.07 (C-5), 73.25 (2-O-CH_2), 75.48 (3-O-CH_2), 79.25 (C-4), 79.81 (C-3), 80.53 (C-2), 97.59 (C-1)

Hexakis (2,3-di-O-butyl)-α-cyclodextrin (10)



$C_{84}H_{156}O_{30}$
EM=1645.07

Yield = 79.3%

Rf=0.38 ($CHCl_3$:MeOH = 97:3)

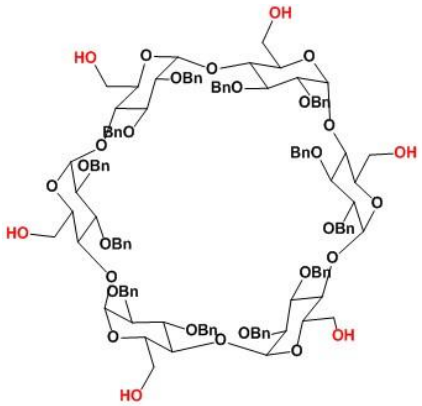
T_m= 227.7 ºC

Mass ESI (m/z)= [$[M+H]^+$ 1645.9, **[**$[M+Na]^+$ 1668.3

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.89-0.93 (m, 36H, 2-O-CH_2-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-CH_2-C*H_3*), 1.29-1.42 (m, 24H, 2-O-CH_2-CH_2-C*H_2*-CH_3, 3-O-CH_2-CH_2-C*H_2*-CH_3), 1.53-1.61 (m, 24H, 2-O-CH_2-C*H_2*-CH_2-CH_3, 3-O-CH_2-C*H_2*-CH_2-CH_3), 3.22 (dd, 6H, H-2, J=3.6Hz and 10Hz), 3.50-3.62 (m, 18H, H-4, H-3, H-5), 3.65-3.73 (m, 24H, 2-O-C*H_2*-CH_2-CH_2-CH_3, 3-O-C*H_2*-CH_2-CH_2-CH_3), 3.97-4.05 (m, 12H, H-6a, H-6b), 5.09 (d, 6H, H-1, J=3.6Hz)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): 14.03 (2-CH_3 (Bu)), 14.14 (3-CH_3 (Bu)), 19.24 (2-O-CH_2-CH_2-*C*H_2-CH_3), 19.25 (3-O-CH_2-*C*H_2-CH_2-CH_3), 32.39 (2-O-CH_2-*C*H_2-CH_2-CH_3), 32.60 (3-O-CH_2-CH_2-*C*H_2-CH_3), 62.44 (C-6), 71.38 (2-O-*C*H_2-CH_2-CH_2-CH_3), 73.03 (C-5), 73.80 (3-O-*C*H_2-CH_2-CH_2-CH_3), 77.23 (C-4), 79.80 (C-3), 80.56 (C-2), 97.54 (C-1)

Hexakis (2,3-di-O-benzyl)-α-cyclodextrin (11)



$C_{120}H_{132}O_{30}$
EM=2052.88

Yield=54.4%

Rf=0.22 ($CHCl_3$:MeOH = 9:0.5)

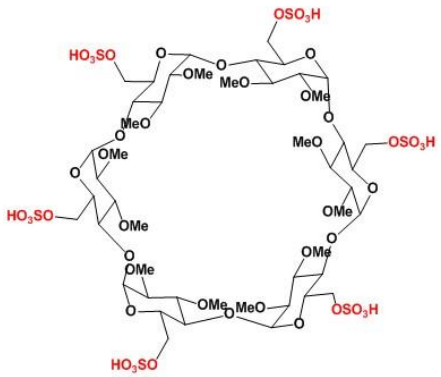
T_m= 146.5 ºC

Mass ESI (m/z)= [$M+H]^+$ 2054.9, **[**$M+Na]^+$ 2076.8

^{1}H NMR ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 3.46 (dd, 6H, H-2, J=3.6Hz and 10 Hz), 3.60-3.68 (m, 12H, H-4, H-6a), 3.85-3.88 (m, 6H, H-6b), 4.02-4.09 (m, 12H, H-3, H-5), 4.42 (d, 6H, H-7a, J=12Hz), 4.58 (d, 6H, H-7b, J=12.4Hz), 4.81 (d, 6H, H-8a, J=10.8Hz), 5.00 (d, 6H, H-1, J=3.2Hz), 5.09 (d, 6H, H-8b, J=10.8Hz), 7.11-7.19 (m, 72H, aromatic)

^{13}C NMR ($CDCl_3$, 100 Hz, characterized by COSY, DEPT): δ (ppm): 62.33 (C-6), 73.07 (2-O-CH_2), 73.25 (C-5), 75.46 (2-O-CH_2), 78.95 (C-4), 79.63 (C-3), 80.84 (C-2), 97.98 (C-1), 127.05-128.24 (C_{ar})

Hexakis (2,3-di-O-methyl-6-O-sulfate)-α-cyclodextrin, DS=6 (12)



$C_{48}H_{84}O_{48}S_6$
EM=1620.25

Yield=100%

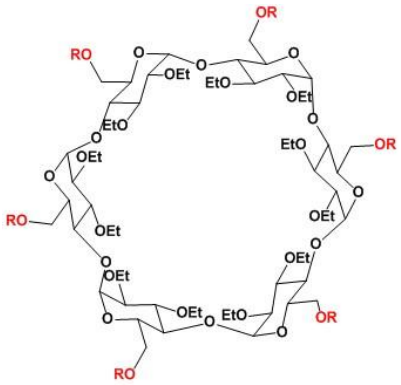
T_m= > 410 ºC

Mass ESI (m/z)= [$M-H]^-$ 1619.2, **[**$M+5H+5Na-H]^-$ 1729.1

^{1}H NMR (D_2O, 400 MHz, characterized by COSY, DEPT): δ (ppm): 3.31 (dd, 6H, H-2, J=3.2Hz and 9.6Hz), 3.50 (s, 18H, 2-O-CH_3), 3.62 (s, 18H, 3-O-CH_3), 3.75-3.84 (m, 12H, H-3, H-4), 4.11 (d, 6H, H-5, J=8.8Hz), 4.26 (d, 6H, H-6a, J=10.8Hz), 4.53-4.55 (m, 6H, H-6b), 5.29 (d, 6H, H-1, J=3.2Hz)

^{13}C NMR (D_2O, 100 Hz, characterized by COSY, DEPT): δ (ppm): 57.76 (2-O-CH_3), 59.91 (3-O-CH_3), 67.29 (C-6), 69.84 (C-5), 78.47 (C-3), 79.79 (C-3), 80.64 (C-2), 97, 38 (C-1)

Hexakis (2,3-di-O-ethyl-6-O-sulfate)-α-cyclodextrin, DS=5.46 (13)



$(C_{12}H_{18}O_5)_6(SO_3)_n$

Yield=73.5%

T_m= 125.6 ºC

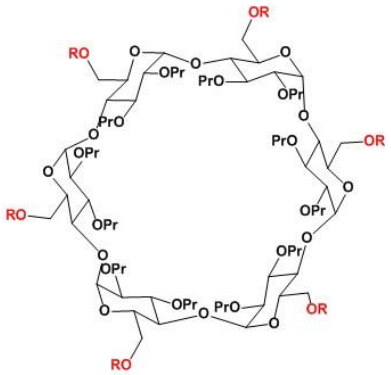
MW= 1309.54 **(**n=1), 1469.61 (n=2), 1549.67 (n=3), 1629.73 (n=4), 1709.80 (n=5), 1789.86 (n=6), **1746.28 (DS=5.46)**

Mass ESI (m/z)= [M-H]$^-$ 1787.4, **[**M-H+Na]$^-$ 1809.4

^{1}H NMR (MeOD, 300 MHz, characterized by COSY, DEPT): δ (ppm): 1.09-1.15 (m, 36H, 2-O-CH_2-C*H*$_3$, 3-O-CH_2-C*H*$_3$), 3.02 (dd, 6H, H-2, J=3.2Hz and 10.1Hz), 3.53-3.58 (m, 6H, H-4), 3.62-3.68 (m, 12H, 2-O-C*H*$_2$-CH_3), 3.73-3.78 (m, 12H, 3-O-C*H*$_2$-CH_3), 3.87-3.98 (m, 12H, H-3, H-5), 4.32 (d, 6H, H-6a, J=11.4Hz), 4.48 (d, 6H, H-6b, J=11.1Hz), 5.31 (d, 6H, H-1, J=3Hz)

^{13}C NMR (D_2O, 100 Hz, characterized by COSY, DEPT): δ (ppm): 14.72 (2-O-CH_2-*C*H_3), 14.75 (3-O-CH_2-*C*H_3), 66.83 (C-6), 67.43 (2-O-*C*H_2-CH_3), 68.57 (3-O-*C*H_2-CH_3), 69.97 (C-5), 77.93 (C-4), 78.15 (C-3), 79.33 (C-2), 97.82 (C-1)

Hexakis (2,3-di-O-propyl-6-O-sulfate)-α-cyclodextrin, DS=5.14 (14)



$(C_{12}H_{22}O_5)_6(SO_3)_n$

Yield=87.9%

T_m= 221.9 ºC

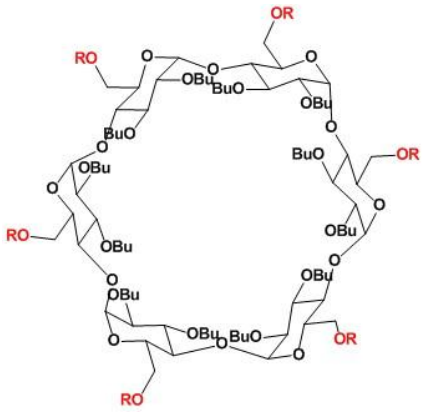
MW= 1557.86 (n=1), 1637.93 (n=2), 1717.99 (n=3), 1798.05 (n=4), 1878.12 (n=5), 1958.18 (n=6), **1889 (DS=5.14)**

Mass ESI (m/z)= [M-H]$^-$ 1955.6

^{1}H NMR (D_2O, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.85-0.94 (m, 36H, 2-O-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-C*H_3*), 1.60-1.66 (m, 24H, 2-O-CH_2-C*H_2*-CH_3, 3-O-CH_2-C*H_2*-CH_3), 3.28 (d, 6H, H-2, J=8.8Hz), 3.54 (d, 6H, H-4, J=7.2Hz), 3.65-3.80 (m, 24H, 2-O-C*H_2*-CH_2-CH_3, 3-O-C*H_2*-CH_2-CH_3), 3.34-3.96 (m, 12H, H-3, H-5), 4.26 (d, 6H, H-6a, J=7.6Hz), 4.69 (d, 6H, H-6b, J=8.4Hz), 5.3 (s, 6H, H-1)

^{13}C NMR (D_2O, 100 Hz, characterized by COSY, DEPT): δ (ppm): 9.70 (2-O-CH_2-CH_2-*C*H_3), 10.10 (3-O-CH_2-CH_2-*C*H_3), 22.74 (2-O-CH_2-*C*H_2-CH_3), 23.04 (3-O-CH_2-*C*H_2-CH_3), 67.14 (C-6), 69.91 (C-5), 72.77 (2-O-*C*H_2-CH_2-CH_3), 76.00 (3-O-*C*H_2-CH_2-CH_3), 77.6 (C-4), 78.81 (C-3), 80.52 (C-2), 97.02 (C-1)

Hexakis (2,3-di-O-butyl-6-O-sulfate)-α-cyclodextrin, DS=5.70 (15)



R= -SO_3H, -H

($C_{14}H_{26}O_5$)$_6$(SO_3)$_n$

Yield=91.4%

T_m= 233.2 ºC

MW= 1726.18 (n=1), 1806.25 (n=2), 1886.31 (n=3), 1966.37 (n=4), 2046.44 (n=5), 2126.50 (n=6), **2102.12 (DS=5.70)**

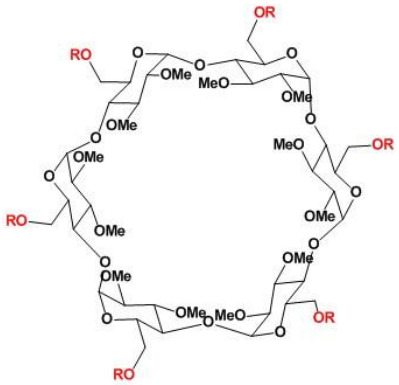
Mass ESI (m/z)= [M-H]$^-$ 2124.8, **[**M-H+Na]$^-$ 2145.8

^{1}H NMR (MeOD, 400 MHz, characterized by COSY, DEPT): δ (ppm): 0.80-0.87 (m, 36H, 2-O-CH_2-CH_2-CH_2-C*H_3*, 3-O-CH_2-CH_2-CH_2-C*H_3*), 1.23-1.36 (m, 24H, 2-O-CH_2-CH_2-C*H_2*-CH_3, 3-O-CH_2-CH_2-C*H_2*-CH_3), 1.44-1.55 (m, 24H, 2-O-CH_2-C*H_2*-CH_2-CH_3, 3-O-CH_2-C*H_2*-

CH_2-CH_3), 3.09 (dd, 6H, H-2, J=3.0Hz and 9.8Hz), 3.50-3.67 (m, 30H, H-3, 2-O-C*H*$_2$-CH_2-CH_2-CH_3, 3-O-C*H*$_2$-CH_2-CH_2-CH_3), 3.74-3.84 (m, 12H, H-4, H-5), 4.24 (d, 6H, H-6, J=11.2Hz), 4.47 (d, 6H, H-6b, J=10.4Hz), 5.30 (s, 6H, H-1)

^{13}C NMR (MeOD, 100 Hz, characterized by COSY, DEPT): δ (ppm): 13.07 (2-CH_3 (Bu)), 13.25 (3-CH_3 (Bu)), 19.00 (2-O-CH_2-CH_2-*C*H_2-CH_3), 19.07 (3-O-CH_2-CH_2-*C*H_2-CH_3), 32.28 (2-O-CH_2-*C*H_2-CH_2-CH_3), 32.42 (3-O-CH_2-CH_2-*C*H_2-CH_3), 67.02 (C-6), 70.15 (C-5), 70.87 (2-O-*C*H_2-CH_2-CH_2-CH_3), 73.45 (3-O-*C*H_2-CH_2-CH_2-CH_3), 77.46 (C-4), 79.86 (C-3). 80.56 (C-2), 96.99 (C-1)

Hexakis (2,3-di-O-methyl-6-O-propanesultone)-α-cyclodextrin, DS= 2.91 (16)



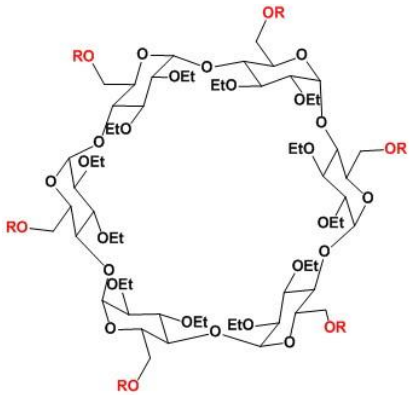
R= -$(CH_2)_3SO_3H$, -H

$(C_8H_{14}O_5)_6(C_3H_6SO_3)_n$

MW= 1263.31 (n=1), 1385.45 (n=2), 1507.59 (n=3), 1629.73 (n=4), 1751.88 (n=5), 1874.02 (n=6), **1501.02 (DS=2.91)**

Mass ESI (m/z)= *6 $(CH_2)_3SO_3$:* [M-H] $^-$1871.5, [M-2H+Na]$^-$ 1893.4; 5 *$(CH_2)_3SO_3$:* [M-H]$^-$ 1751.5; *4 $(CH_2)_3SO_3$:* [M-H]$^-$ 1627.5; [M-2H+Na]$^-$ 1649.4; *3 $(CH_2)_3SO_3$*: [M-H]$^-$ 1505.5; [M-2H+Na]$^-$ 1527.5; *2 $(CH_2)_3SO_3$*: [M-H]$^-$ 1383.5; *1 $(CH_2)_3SO_3$*: [M-H]$^-$ 1261.5

Hexakis (2,3-di-O-ethyl-6-O-propanesultone)-α-cyclodextrin, DS=4.64 (17)



R= -$(CH_2)_3SO_3H$, -H

$(C_{10}H_{18}O_5)_6(C_3H_6SO_3)_n$

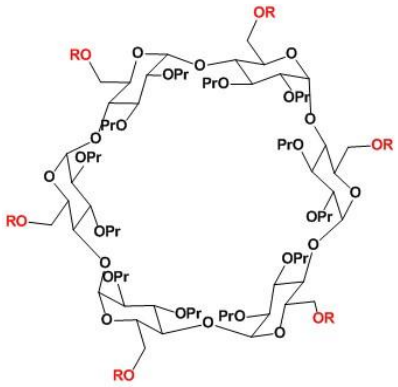
T_m= 299.2-332.6 °C

MW= 1431.62 (n=1), 1553.77 (n=2), 1675.91 (n=3), 1798.05 (n=4), 1920.20 (n=5), 2042.34 (n=6), **1875.56 (DS=4.64)**

Mass ESI (m/z)= *6 $(CH_2)_3SO_3$:* $[M-3H]^{3-}$ 679.2, $[M-2H]^{2-}$ 1019.4; 5 *$(CH_2)_3SO_3$:* $[M-3H]^{3-}$ 638.9, $[M-2H]^{2-}$ 958.4; *4 $(CH_2)_3SO_3$:* $[M-3H]^{-3}$ 597.9, $[M-2H]^{2-}$ 897.4; *3 $(CH_2)_3SO_3$*: $[M-H]^-$ 1673.7, *2 $(CH_2)_3SO_3$*: $[M-H]^-$ 1551.7; *1 $(CH_2)_3SO_3$*: $[M-H]^-$ 1429.7

NMR ^{1}H ($CDCl_3$, 400 MHz, characterized by COSY, DEPT): δ (ppm): 1.21-1.23 (m, 36H, 2-O-CH_2-C*H_3*, 3-O-CH_2-C*H_3*), 2.01-2.06 (m, 12H, CH_2-CH_2-C*H_2*-SO_3), 2.83-2.91 (m, 12H, CH_2-C*H_2*-CH_2-SO_3), 3.22-3.26 (m, 6H, H-2),3.50-3.78 (m, 66H, H-3, H-4, H-5, H-6a, 2-O-C*H_2*-CH_3, 3-O-C*H_2*-CH_3, C*H_2*-CH_2-CH_2-SO_3), 4.04-4.16 (m, 6H, H-6b), 5.07-5.36 (m, 6H, H-1)

Hexakis (2,3-di-O-propyl-6-O-propanesultone)-α-cyclodextrin, DS=4.19 (18)



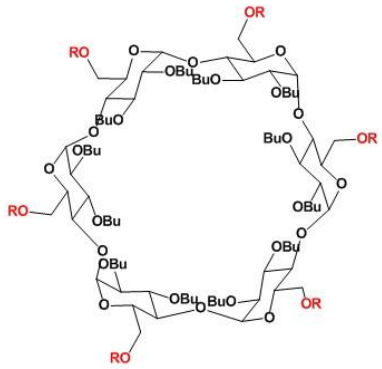
R= -$(CH_2)_3SO_3H$, -H

$(C_{12}H_{22}O_5)_6(C_3H_6SO_3)_n$

MW= 1599.94 (n=1), 1722.09 (n=2), 1844.23 (n=3), 1966.37 (n=4), 2088.52 (n=5), 2210.66 (n=6), **1988.98 (DS=4.19)**

Mass ESI (m/z)= 3 *$(CH_2)_3SO_3$:* $[M-H]^-$ 1841.9; 4 *$(CH_2)_3SO_3$:* $[M-H]^-$ 1963.9; 5 *$(CH_2)_3SO_3$:* $[M-H]^-$ 2086.9

Hexakis (2,3-di-O-butyl-6-O-propanesultone)-α-cyclodextrin, DS= 3.84 (19)



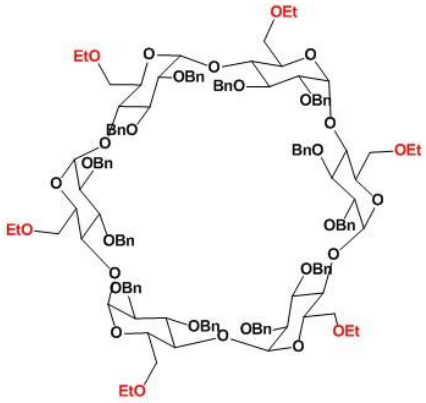
R= $-(CH_2)_3SO_3H$, -H

$(C_{14}H_{26}O_5)_6(C_3H_6SO_3)_n$

MW= 1769.26 (n=1), 1890.41 (n=2), 2012.55 (n=3), 2134.69 (n=4), 2256.83 (n=5), 2378.98 (n=6), **2114.6 (DS=3.84)**

Mass ESI (m/z)= *6 $(CH_2)_3SO_3$:* $[M-4H]^{-4}$ 593.2; 5 *$(CH_2)_3SO_3$:* $[M-2H]^{2-}$ 1127.2, $[M-3H]^{3-}$ 751.0; *4 $(CH_2)_3SO_3$:* $[M-2H]^{2-}$ 1066.0, $[M-3H]^{3-}$ 710.4; *3 $(CH_2)_3SO_3$:* $[M-2H]^{2-}$ 1005.0, $[M-3H]^{3-}$ 669.7

Hexakis (2,3-di-O-benzyl-6-O-ethyl)-α-cyclodextrin (20)



$C_{132}H_{156}O_{30}$
EM=2221.07

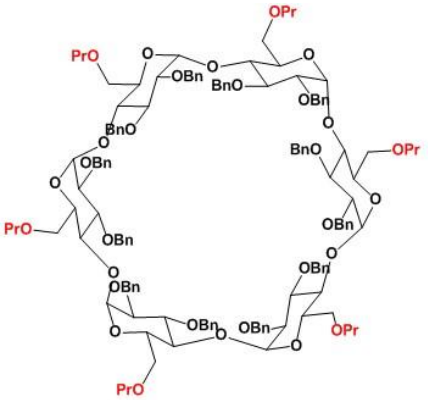
Yield=85%

Rf=0.84 (DCM:MeOH=98:2)

Mass ESI (m/z)= [$M+H]^+$ 2223.0, **[**$M+Na]^+$ 2245.0

NMR ^{1}H ($CDCl_3$, 300 MHz): δ (ppm): 1.14 (t, 18H, C*H*$_3$, J=7.5Hz), 3.38-3.59 (m, 24H, H-2, H-3, 6-O-C*H*$_2$), 3.90-3.94 (m, 18H, H-4, H-5, H-6a), 4.1-4.15 (m, 6H, H-6b), 4.39-4.51 (q, 12H, H-7a, H-7b, J=12Hz), 4.88 (d, 6H, H-8a, J=12Hz), 5.03 (d, 6H, H-1, J=3Hz), 5.20 (d, 6H, H-8b, J=9Hz), 7.13-7.27 (m, 72H, aromatic)

Hexakis (2,3-di-O-benzyl-6-O-propyl)-α-cyclodextrin (21)



$C_{138}H_{168}O_{30}$
EM=2305.16

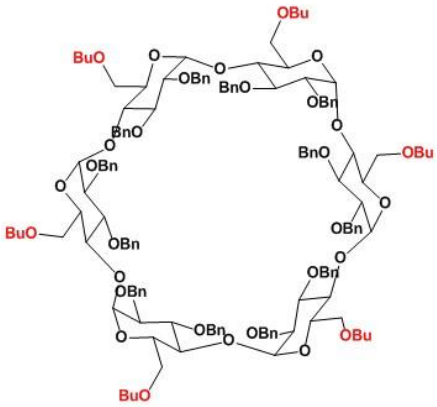
Yield=84%

Rf=0.87 (DCM:MeOH=98:2)

Mass ESI (m/z)= [M+H]$^+$ 2307.1

NMR ^{1}H (CDCl$_3$, 300 MHz): δ (ppm): 0.99 (t, 18H, C*H$_3$*), 1.64-1.71 (m, 12H, C*H$_2$*-C*H$_3$*), 3.40-3.69 (m, 24H, H-2, H-3, 6-O-C*H$_2$*), 4.01-4.11 (m, 18H, H-4, H-5, H-6a), 4.25 (t, 6H, H-6b, J=7.5Hz), 4.59 (q, 12H, H-7a, H-7b, J=12Hz), 4.99 (d, 6H, H-8a, J=9Hz), 5.22 (d, 6H, H-1, J=3Hz), 5.30 (d, 6H, H-8b, J=9Hz), 7.24-7.37 (m, 72H, aromatic)

Hexakis (2,3-di-O-benzyl-6-O-butyl)-α-cyclodextrin (22)



$C_{144}H_{180}O_{30}$
EM=2389.26

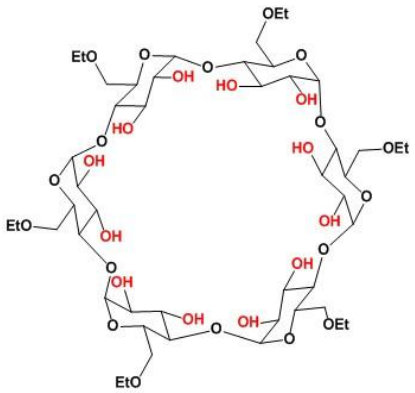
Yield=34%

Rf=0.61 (DCM:MeOH=99:0.5

Mass ESI (m/z)= [M+H]$^+$ 2391.2, **[**M+Na]$^+$ 2413.1

NMR ^{1}H (CDCl$_3$, 300 MHz): δ (ppm): 0.88 (t, 18H, C*H$_3$*, J=7.5Hz), 1.26-1.38 (m, 12H, C*H$_2$*-CH$_3$), 1.48-1.55 (m, 12H, 6-O-C*H$_2$*), 3.30-3.54 (m, 24H, H-2, H-3, 6-O-C*H$_2$*), 3.88-4.02 (m, 12H, H-4, H-5, H-6a), 4.13 (t, 6H, H-6b, J=9Hz), 4.48 (q, 12H, H-7a, H-7b, J=15Hz), 4.87 (d, 6H, H-8a, J=9Hz), 5.12 (d, 6H, H-1, J=3Hz), 5.18 (d, 6H, H-8b, J=9Hz)

Hexakis (6-O-ethyl)-α-cyclodextrin (23)



$C_{48}H_{84}O_{30}$
EM=1140.50

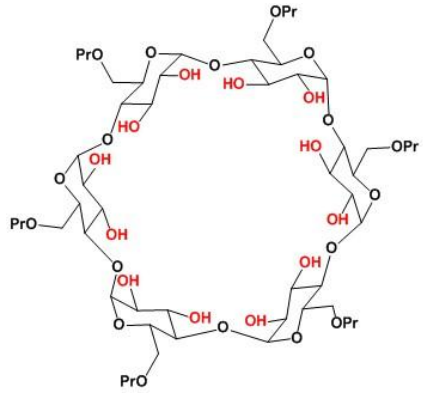
Yield=37%

Rf=0.26 ($CHCl_3$:MeOH = 8:2

Mass ESI (m/z)= [$[M+H]^+$ 1141.5, **[**$[M+Na]^+$ 1163.5

NMR ^{1}H ($CDCl_3$, 300 MHz): δ (ppm): 1.19-1.25 (m, 18H, C*H*$_3$), 3.53-3.58 (m, 18H, H-2, O-C*H*$_2$), 3.71-3.76 (m, 18H, H-3, H-4, H-5), 3.97-4.05 (m, 12H, H-6a, H-6b), 4.95 (d, 6H, H-1. J=3Hz)

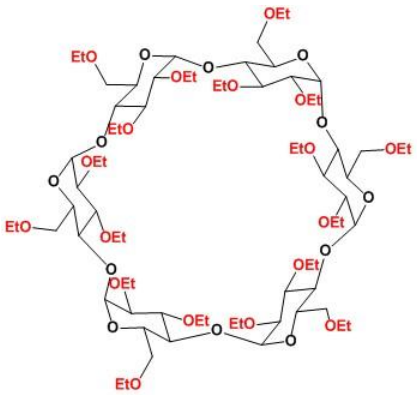
Hexakis (6-O-propyl)-α-cyclodextrin (24)



$C_{54}H_{96}O_{30}$
EM=1224.60

Mass ESI (m/z)= [$[M+Na]^+$ 1247.5

Hexakis (2,3,6-tri-O-ethyl)-α-cyclodextrin (25)



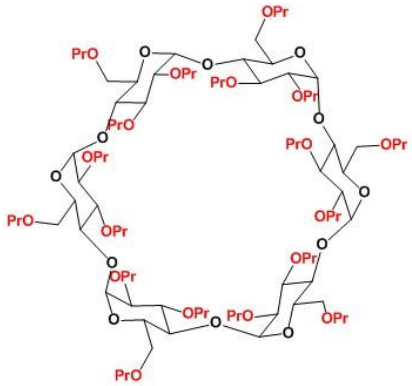
$C_{72}H_{132}O_{30}$
EM=1476.88

Rf=0.36 ($CHCl_3$:MeOH = 99:1)

Mass ESI (m/z)= [$M+H]^+$ 1477.8, **[**$M+Na]^+$ 1500.0

NMR ^{1}H ($CDCl_3$, 300 MHz): δ (ppm): 0.52-0.58 (m, 54H, C*H*$_3$), 2.50 (dd, 6H, H-2, J=6Hz and 9Hz), 2.73-2.92 (m, 42H, H-3, O-C*H*$_2$), 3.09-3.15 (m, 12H, H-4, H-5), 3.31-3.49 (m, 12H, H-6a, H-6b), 4.37 (d, 6H, H-1, J=6Hz)

Hexakis (2,3,6-tri-O-propyl)-α-cyclodextrin (26)



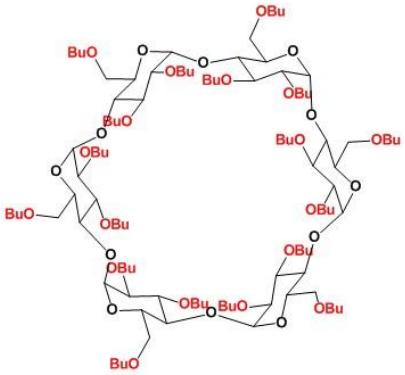
$C_{90}H_{168}O_{30}$
EM=1729.16

Rf=0.37 (cyclohexane:diethyl ether=7:3)

Mass ESI (m/z)= [$M+H]^+$ 1730.1, **[**$M+Na]^+$ 1752.1

NMR ^{1}H ($CDCl_3$, 300 MHz): δ (ppm): 0.85-0.93 (m, 54H, C*H*$_3$), 1.55-1.64 (m, 36H, C*H*$_2$), 3.23 (dd, 6H, H-2, J=3Hz and 9Hz), 3.29-3.35 (m, 6H, H-3), 3.45-3.74 (m, 48H, H-4, H-5, O-C*H*$_2$), 3.98-4.02 (m, 12H, H-6a, H-6b), 5.18 (d, 6H, H-1, J=3Hz)

Hexakis (2,3,6-tri-O-butyl)-α-cyclodextrin (27)



$C_{108}H_{204}O_{30}$
EM=1981.44

Rf=0.67 (cyclohexane:diethyl ether=7:3)

Mass ESI (m/z)= [$M+H]^+$ 1983.4, **[**$M+Na]^+$ 2005.3

B. Calixarenes

1. MTT assay

For C4S, a cell viability of 100% was maintained to 1mM and then decreased to 0% at 5mM. With regard to C6S, the behaviour was very different, as 0% viability was observed at only 2mM. It should be noted that the purity of the compound was verified. For C8S, 100% cell viability was maintained until 1mM, and decreased to 0% at 5mM. (Figure 33.) [274]

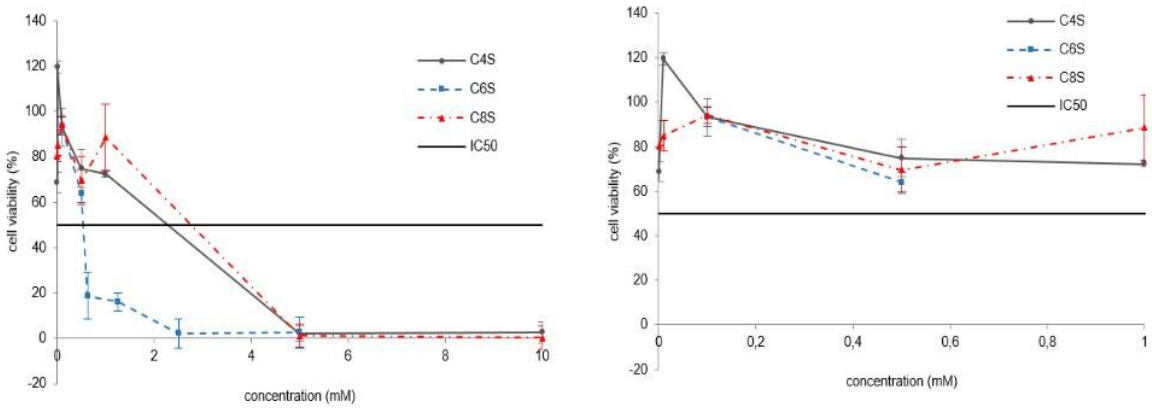


Figure 33 *MTT test on the Caco-2 cells in the presence of three para-sulphonato-calix[n]arenes, over the concentration range of 1–10mM (left) and over the concentration range above the* IC_{50} *values (right).*

2. Transport study

A very clear difference is observed between C4S and C8S on one hand and C6S on the other hand. For C6S there is no passage of LY across the Caco-2 membrane neither with pre-treatment nor with simultaneous treatment. (Figure 34) For C4S, no passage is induced by pre-treatment, however after an induction period of 20 min transport commences giving 1µg detected after 90min. Above this time passage increases sharply to about 3.5µg after 120min. The behaviour of C8S is generally similar, with no passage after pre-treatment. However, for simultaneous application there is a small but real passage in the 5min to 30min zone and an effectively linear increase until 120min where passage of about 2.8µg is observed. [274]

Concerning TEER measurements; when pre-treatment with *para*-sulphonato-calix[n]arenes was used, the TEER value remained above 1000Ωxcm^2, with no significant difference from the starting value, showing that the calix[n]arenes do not degrade the Caco-2 monolayer structure. For the combined transport experiments using the *para*-sulphonato-calix[n]arenes

and LY together, the TEER value seriously decreased to under 200Ωxcm^2 for C4S and C8S and around 600Ωxcm^2 for C6S compared to the starting value above 1000Ωxcm^2. [274]

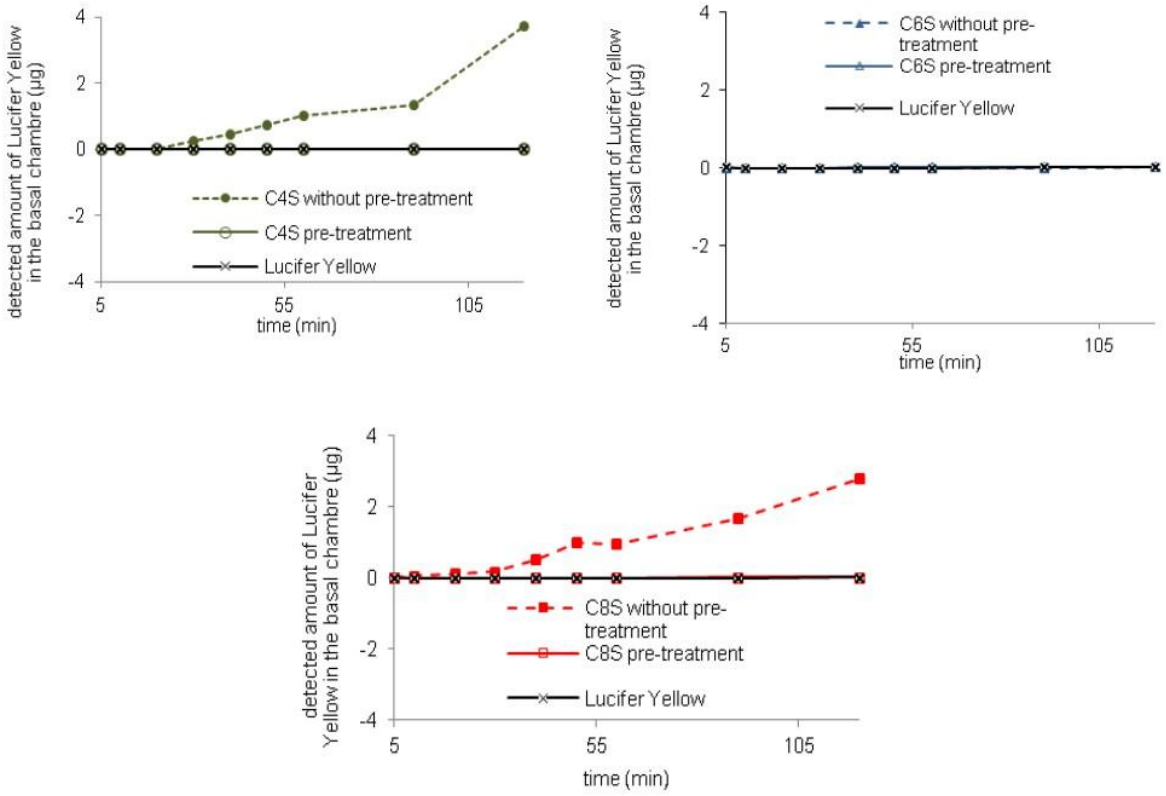


Figure 34 *Transport of LY across the confluent Caco-2 monolayer using para-sulphonato-calix[n]arenes as transporter molecules, the Lucifer Yellow control is carried out in the absence of para-sulphonato-calix[n]arenes but with the same LY concentrations as those used in the combined transport experiments.*

XII. Discussion

A. α-cyclodextrins

In our study, the toxic effects of various α-CD derivatives were investigated *in vitro*. In the pharmaceutical industry, CDs are used as solubility, thus bioavailability enhancer excipients, and several CD-based formulations are available on the market. Native β-CD and its derivatives are the most frequently used, meanwhile α- and γ-CDs are somehow less favoured. This recent generality results from two main factors. The first is that β-CDs have an inner cavity dimension which is accessible for most drug molecules, compared to the two other CDs. The smaller cavity of α-CD limits the range of suitable guest molecules, and the bigger diameter of γ-CD results in loose host-guest interactions, thus less stable inclusions are formed. The preferable size of β-CDs led to the belief of a more potent pharmaceutical utility, thus their general evaluations, including the toxicological description, gained priority over the two other CD families.

Our curiosity was awakened by the growing interest in α-CD utilization, due to its favourable effect on blood lipids and weight loss. On the other hand, as similar chemical modifications have been done on α- and β-CDs, we presumed that the comparison of their *in vitro* behaviour may provide indispensable information on structure-activity correlations. Our work provided information on three levels:

- effect of certain substituents on the biocompatibility, compared to the parent molecule,
- understanding the influence of the number of building units on the toxic profile,
- evaluation of each derivative's effects in three different systems.

In vitro tests were chosen by the endeavour to model the *per os* and intravenous administration routes. As well known, both orally or *i.v.* administered drugs must comply with the crucial criteria of good aqueous solubility. When it can only be provided in the presence of excipients (e.g. CDs), the throughout evaluation of their behaviour in a biological system is essential. It has to be clarified, whether the CD has a disadvantageous effect on the endothelium, thus the enhanced absorption only results from the disruption of the barrier, or we face indeed an advantageous bioavailability enhancer effect. Furthermore, when they are administered intravenously, the possibility of hemolysis must be excluded. Our work targets the limitation of adverse effects in human, by a reliable guide for the determination of a safe concentration range for those α-CDs, which have a potential for future pharmaceutical applications.

Tests were performed on Caco-2 cells and on human erythrocytes. IC_{50} values were determined by both MTT assay and RT-CES method, and HC_{50} by hemolysis test. MTT assay is based on the enzymatic conversion of MTT dye in the mitochondria, and detects early cytotoxicity compared to the two other methods [275]. Hemolysis test was a simple and rapid investigation process to range the CDs according to their haemolytic activity [232]. Both methods are so called end-point tests, in the sense that they visualize if cell death/lysis befell, but do not provide kinetic data. [276] RT-CES does not only support the determination of toxic concentrations, but as the assay is performed for the whole length of the experiment, it characterizes the kinetic factors of toxic processes. [207] The resulting data are biologically relevant, because the elimination of labels brings the cells closer to the physiological conditions. Ozsvári *et al.* observed similar results of cytotoxicity using immortalized and rat primary cell lines in RT-CES experiments. [207] The correlation between *in vitro* IC_{50} and *in vivo* LD_{50} values was verified by Boyd *et al.* [277] RT-CES provides reliable and dynamic cytotoxic parameters. This method was the most sensitive technique in our study.

The influence of the nine α-CD derivatives on Caco-2 cell viability, and the their hemolytic evaluation led us to four general conclusions, as follows:

1. **Chemical modification on the free hydroxyl groups have a definite impact on toxicity.** In each case, a structural modification resulted change in both the IC_{50} and HC_{50} values, compared to the parent molecule. Both methods performed on Caco-2 cells indicates, that the presence of three methyl groups per glucopyranose residue (TRIMEA), phosphate groups (phosphated ACD) or succinyl groups (SuACD) resulted in lower IC_{50} values, thus increased the toxicity. On the other hand, hydroxypropylation (HPACD) and acetylation (AcACD) caused such a reduction, that their IC_{50} values could have not been determined up to 100mM. [273]
2. **Toxicity depends on the number of building units.** The IC_{50} and HC_{50} value of those α- and β-CD derivatives, which have undergone the same chemical modifications, were correlated to their parent molecules, and then compared. We have discovered that a certain substitution pattern may have contrary effect, meanwhile others shifted the toxic profile similarly. For example, hydroxypropylation decreases the toxicity: neither HPACD, nor HPBCD has a ponderable IC_{50} value up to 100mM and 200mM, respectively. Although, HPBCD is hemolytic from 57mM, but HPACD is safe to use up to 100mM. [6] On the other hand, TRIMEA proved to have more severe cytotoxic and hemolytic effect than

RAMEA, while RAMEB has a lower IC_{50} and HC_{50} concentration (30mM and 1.8mM, respectively) than TRIMEB (45mM and 5.0mM, respectively). [6]

3. **The rate of toxicity depends on the exposition time.** In general, it can be stated that longer exposition cause lower IC_{50} values, thus more severe toxic effect. [273] Apart from HPACD and AcACD, which were non-toxic in all systems, lower IC_{50} concentrations were determined by RT-CES, than by MTT assay. The main difference is the prolonged exposition time, and the permanent contact between the cells and CDs along the RT-CES experiment. For instance, RAMEA showed toxic effect from 78mM with MTT test, but with RT-CES from 25mM. In case of sulfated ACD, no IC_{50} was defined by MTT, but by RT-CES it was determined from 10mM.

4. **The intensity of CD cytotoxicity varies on different cell types**. The rate of increase/decrease in the toxic effect of most derivatives, compared to the parent molecule, differs on Caco-2 cells and on RBCs. This difference is due to the distinct membrane composition of the two cell types. HPACD and AcACD are the exceptions, regarding their non-toxic behaviour in each case. However, while phosphated ACD proved to be toxic on Caco-2 cells, it did not have effect on RBCs. RAMEA had relatively high IC_{50} value, but its hemolytic effect occurred already at 15mM. [273] It is true in general, that HC_{50} values are lower than IC_{50} concentrations, which may indicate that α-CD derivatives have higher affinity to the erythrocyte membrane than to the intestinal cell membranes. Matilainen *et al.* have reported that hydroxypropylated CDs and native γ-CD seemed to be the safest, and RAMEB was the least safe on lung cells (Calu-3). [278] Kiss *et al.* found the following order regarding the cytotoxicity of β-CDs on Caco-2 cells: RAMEB > TRIMEB > DIMEB > PARMEB > HPBCD ~ CMBCD. [6] Interestingly, the cytotoxicity of α-CD towards human corneal epithelial cells was even greater than of DIMEB, to which a relatively low IC_{50} value belongs. [279] These results all confirm that the intensity of cytotoxicity depends on the cell, thus membrane types. As every cell type has a unique membrane structure, a certain CD derivative disrupts their integrity in different rates.

Accurate structure-activity correlations were found when CD derivatives with slight structural differences were compared by MTT assay. Namely, those of the methylated CDs which possess a distinct number of substituents, showed significant differences in cytotoxicity. Increasing number of methyl groups resulted lower IC_{50} values, thus elevating toxicity. When the hydroxyl groups at each position were modified, thus no free hydroxyls were left at the CD ring (TRIMEA), IC_{50} was determined at a rather low concentration (1.8mM). DIMEA, RAMEA

and PARMEA possessing 12, ~11 and ~9 methyl groups, respectively, proved to have toxic effect at elevating concentrations. These observations led to two main conclusions. Firstly, increasing number of methyl groups presented on the α-CD ring has no beneficial effect on CD toxicity. While DIMEA had similar effect to the parent molecule, only RAMEA and PARMEA reduced the cytotoxicity of the native α-CD. Thus, to improve the toxic profile of native α-CD, maximum 11 hydroxyl groups shall be modified, at least by methylation. This consideration drove to the second statement, which confirms the necessity of free hydroxyl groups for the amelioration of the cytotoxic profile.

Derivatives **12** and **13** have a sulfate group at the C-6 position. They differ in the length of the alkyl group at positions C-2 and C-3. **12** have 12 methyl, **13** has 12 ethyl groups at the secondary face. The DS for the sulfate substituent is 6 in case of **12**, and slightly less, 5.5 for **13**. Compared to the parent molecule (DIMEA), the cytotoxicity of **12** increased. It can be explained by the lack of free hydroxyl groups, also by the effect of the sulfate groups. It has to be noted, that the parent molecule of **13** could have not been tested due to the insolubility of 2,3-di-O-ethyl αCD (compound **8**) in aqueous environment. **13** had more satisfactory toxic profile, as it has a higher IC_{50} value. This effect can be the result of the two excess methylene groups per glucopyranose unit. However, as the DS for sulfate was not exactly 6 but lower, it implies the presence of a few free hydroxyl groups at the primary face; and as it was explained above, the presence of free hydroxyl groups has a highly beneficial vital effect *in vitro*.

Significant correlation was observed between the cytotoxicity determined by MTT on Caco-2 cells, the hemolytic activity and the cholesterol complexation capacity of various β-CDs. [6] β-CDs can extract cholesterol from the lipid rafts and RAMEB can enter the intestinal epithelial cells by endocytosis. [7] The mechanism of α-CD cytotoxicity is different, because its cavity is too small to include a cholesterol molecule. The acyl chain of phospholipids, however, fits well within the α-CD cavity. [230, 231] In our indirect verification, the RAMEA-phosphatidylcholine complex did not result in toxicity on Caco-2 cells, compared to the unloaded RAMEA. It proves, that toxic effect does not occur when the CD cavity is in complex. However, phosphatidylcholine extraction alone does not explain the toxic behaviour. Phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and sphingomyelin also shows affinity for α-CDs. [231] Phospholipids are present in the external and internal layers of RBC membrane in different ratios. [231] This explains the concentration dependent hemolytic effect of α-CDs. The most severe hemolytic activity was shown by TRIMEA and SuACD, in accordance with the MTT results. HPACD and AcACD had diminished hemolytic activity

(HC_{50}>100mM). Our results are in correlation with the findings of Leroy-Lechat *et al.*, namely that hydroxypropylation lowers the hemolytic activity in case of α-, β- and also γ-CD. [232] All authors, including us, agree with Irie *et al.* and Bost *et al.* [179, 280]: the complexation of the membrane components induces the lysis of the erythrocytes or the irreversible damage of Caco-2 cells.

Synthetic work

The fact has been accepted, that most of the commercially available CD derivatives are mixtures. [64] The DS value gives useful information about the approximate structure, however one batch is composed of molecules with different substitution pattern. The importance of the number of substituents has been already shown by Irie and Uekama. [179, 281] They reported the different hemolytic activity of the methylated and hydroxypropylated β-CDs, which differ in the DS. Mosher and Thompson have published the dramatic difference found between the hemolytic effect of SBEBCDs, having 1, 4 or 7 substituents per CD ring. [181] SBE1BCD already induced hemolysis at higher concentration than the parent molecule, SBE4BCD had significantly milder, while SBE7BCD practically had no hemolytic effect.

Our aim was to synthetize alkyl ether α-CD derivatives, which differ in the position and the length of the alkyl substituents. Modifications were made on the primary, secondary or both faces, solely focused on per-substitutions. The introduced alkyl chains consisted of one (methyl) to four (butyl) carbon atoms. In case of primary and secondary face per-substitutions, the 'long' reaction way was used, thus protective groups were used to avoid the over-substitution. Complete per-modification had been facilitated by using the 'sledgehammer' way, thus when reactants were added in high equivalents, and the substitution at each position were indiscriminate.

Secondary face substitutions resulted derivatives with twelve methyl, ethyl, propyl or butyl groups at the C-2 and C-3 positions. Despite of the presence of six free hydroxyls at the primary face, the aqueous solubility had been significantly decreased as the number of methylene groups increased within the side chains. Only the methyl substitution resulted a water soluble product. The other di-alkyl derivatives could have been dissolved only in the presence of an organic co-solvent, such as EtOH or DMSO (e.g. 30%, 84% and 84% EtOH referring to compound **8**, **9** and **10**, respectively), which hamstrung the *in vitro* evaluations. In order to overcome the problem of solubility, further structural changes were carried out. The primary hydroxyls were modified with hydrophile anionic substituents, namely sulfate or sulfopropyl

groups. Both type of changes resulted in high aqueous solubility. The DS values related to the sulfate groups were significantly higher (6-5.1) than those of related to the sulfopropyl groups (4.6-2.9). Among sulfate derivatives, complete substitution could have been achieved on the methylated CD, but the DS of sulfates decreased as the alkyl chain lengthened. Sulfopropylation has put two obstacles in the way. Firstly, the DS values turned to be relatively low. Secondly, the purification procedure became painstaking, despite of the application of several techniques. The origin of this problem was the probable inclusion of sulfopropyl derived salts within the CD cavity.

Primary face alkylation has combined five reaction steps, which have the benefit of excluding alkylation at the secondary phase. Even though the individual steps can give a high yield, the main drawback of this synthesis was the really small overall yield, which hampered the further evaluations. Complete per-substitution provided similar challenges. The 'sledgehammer' way resulted in a mixture of derivatives bearing 1-18 alkyl groups. Due to the slight physicochemical differences between them, their chromatographic separation was particularly cumbersome, and the separated final product had often very small yield. Moreover, complete per-alkylation resulted products with poor water solubility, due to the lack of free hydroxyl groups. However, different methodologies are also described in the literature aiming the production of the above mentioned derivatives.

B. Calixarenes

The seemingly beneficial sulfonation of CDs (regarding both solubility and toxicological profile) drove us to the similarly modified representatives of calixarenes. The focus of our attention was on the evaluation of their effects in Caco-2 cell systems.

It became evident quite soon, that the unique structure is not the only advantage of calix[n]arenes, but they deliver additional benefits regarding their versatile biological activity (e.g. antituberculous, antibiotic, antiviral, anti-thrombotic activity, etc.) [238] Another improvement compared to CDs is their negligible hemolytic effect. For all CnS derivatives which were tested by *Coleman et al*., maximum 5% of hemolysis was observed up to 50mM. [266] The toxicological profile of various calix[n]arenes in different *in vitro* systems has already been reported, and the results are unambiguously promising. Their mutagenic effect was excluded by Ames test [264], they did not affect the growth of human fibroblasts [238], neither activated neutrophil granulocytes. [265]

However, to our best knowledge, *para*-sulphonato-calix[n]arenes have been scarcely investigated from the point of view of their effect on paracellular absorption from the small intestine. We found it crucial to establish these effects, as it represents an important step in their future application as transporter systems. However, calix[n]arenes are not yet approved by the FDA for pharmaceutical use, but their potential has already been demonstrated.

Our aim was to evaluate the effects of three calix[n]arene sulfonates (n=4, 6, 8) on paracellular absorption. In the first place, the IC_{50} concentration was defined for all derivatives on Caco-2 cells by MTT assay. Surprisingly, in each case, IC_{50} concentrations were quite low, thus their negative effect on cell viability had an early occurrence. [274]

In the transport study, LY was used as the paracellular marker. The effect of *para*-sulphonato-calix[n]arenes were tested in two different experiments. First, the cell monolayer was pre-treated with the CnSs and the LY absorption was measured after. In the second case, CnS and LY was applied simultaneously. C6S did not enhance the paracellular transport of LY neither with, nor without pre-treatment. Interestingly, the behaviour of C4S and C8S were similar: no passage was induced by pre-treatment, but when they were combined with LY, there was a detectable enhancement of LY transport.

In case of each pre-treatment, the TEER value remained above $1000\Omega xcm^2$ with no significant change compared to the starting value. Simultaneous application of the used materials manifested in serious TEER reduction in each case. [274]

The difference between C4S and C8S on one hand and C6S on the other is of interest, and suggests that there may be a different mode of action with Caco-2 confluent monolayers, which is in agreement with a difference in the effect on TEER of C4S and C8S compared to the effect of C6S. Of particular interest is the requirement for the combined use of the *para*-sulphonato-calix[n]arenes and LY to induce transport. These differences have provided us a guidance for further evaluations, which aims the better understanding of the underlying mechanism.

C. Novelty and practical relevance of the work

Cyclodextrins

- Chemical modifications on the α-CD ring may reduce (e.g. hydroxypropylation, acetylation, methylation) or enhance (e.g. phosphorylation, succinylation) the toxic behaviour compared to the native parent molecule. [273]
- Similar structural changes (e.g. methylation) may result in contrary effects on α- and β-CD rings. [273]
- Methylation decreases the toxicity depending on the number of substituent groups, but complete per-methylation increases the cytotoxicity. [*unpublished work*]
- The presence of free hydroxyl groups is possibly required for the elaboration of a safe toxic profile. [*unpublished work*]
- α-CDs cause hemolysis on RBCs at lower concentrations, while their undesirable effect on Caco-2 cells arise only at higher concentrations. [273]
- RT-CES showed that α-CD toxicity increases with the exposition time. [273]
- Both hydroxypropylation and acetylation result α-CD derivatives with reduced toxicity, which could be possibly applied even in parenteral formulations. [273]

Calixarenes

- Sulfonation of calix[n]arenes does not only result in better bioavailability, but seems to enhance paracellular absorption. [274]
- *Para*-sulphonato-calix[n]arenes (n=4,6 or 8) have no effect on paracellular absorption when the cell monolayer is treated with calixarenes prior to the addition of the marker molecule. [274]
- C4S and C8S increase the paracellular absorption of Lucifer Yellow when they simultaneously present with the marker molecule. [274]
- C6S did not affect the paracellular absorption in neither case. [274]
- The difference in paracellular absorption enhancement suggests distinct mechanism of calixarenes depending on the ring size. [274]

Summary

The low solubility of drug candidates cause a major problem in pharmaceutical formulations, as the aqueous solubility is an indispensable criterion for appropriate bioavailability. Macrocyclic compounds possess a relatively hydrophobic cavity, which is suitable for guest molecule inclusion. Cyclodextrins and calixarenes are widely studied organic host-compounds, and CDs have already been used as pharmaceutical excipients for solubility enhancement. The macrocycles' chemical structure allows their versatile modification, which eventuates changes not only in physicochemical characteristics, but in their effects on living organisms, as well. Thus, the biocompatibility evaluation of the derivatives is fundamental.

Owing to the already performed assessment of numerous β-CD derivatives' biocompatibility, the aim of this research was to extend these experiments to commercially available α-CDs. They have been used less frequently, however several derivatives, which have not been tested yet *in vitro,* have the possibility of future pharmaceutical use. Their importance is also certified by their benefits in nanoparticle formation. We have been interested in concrete structure-toxicity correlations, thus alkyl ether α-CD derivatives were synthetized bearing increasing length alkyl chains, in different positions. *Para*-sulphonato-calix[n]-arenes have already been widely examined due to their efficient drug complexation and versatile biological activity, however, their effects on paracellular transport mechanism have not been evaluated until now.

The cell viability and hemolysis tests have allowed us to rank the α-CDs and to choose the safest derivatives, also to compare their toxic effects in different systems. The comparison of α- and β-CDs bearing the same chemical modifications highlighted the importance of the number of building units. Important information has been evaluated regarding the connection between the cytotoxic effect and the number of free hydroxyl groups. Derivatives with long alkyl chains possess low solubility, which led us towards further chemical modifications. Sulfonation seemed to have beneficial impact on the biocompatibility. Sulfonation also improved the solubility of calixarenes. C4S and C8S proved their positive effect on paracellular absorption in a non-toxic concentration range, however C6S had no similar effect, thus their behaviour in *in vitro* absorption model system arose forward-looking questions.

Our research concludes, that the structural changes on the macrocyclic rings may have major impact on the biocompatibility. As the modification possibilities are practically unlimited, the evaluation of structure and activity cannot be avoided, facilitating the safest choice for further pharmaceutical use.

Összefoglalás

A gyógyszerhatóanyagok rossz vízoldékonysága nagy kihívást jelent formulálásuk során, ugyanis a vízoldékonyság elengedhetetlen feltétele a megfelelő biohasznosulásnak. A makrociklusos vegyületek belső ürege viszonylag hidrofób, ez alkalmassá teszi őket vendégmolekulákkal való komplexképzésre. A ciklodextrinek és kalixarének széles körben tanulmányozott vegyületek, egyes CD-ek bejegyzett oldékonyságnövelő segédanyagok. A makrociklusok felépítése számos kémiai módosításra ad lehetőséget, amelyek nem csupán a fiziko-kémiai tulajdonságok változását eredményezik, hanem az élő organizmusokra kifejtett hatásokat is módosítják. Ezen származékok biokompatibilitás vizsgálata tehát elengedhetetlen.

Számos β-CD származék biokompatibilitása ismert már, így kutatásunk célul tűzte ki ezen vizsgálatok α-CD-ekre történő kiterjesztését. Az α-CD-ek alkalmazása ritkább, azonban vannak származékok, amelyek *in vitro* vizsgálata még nem történt meg, de jelentőségük a nanopartikulum-képzésben már igazolt. A szerkezet-toxicitás összefüggések feltárása érdekében olyan alkil-éter CD származékokat szintetizáltunk, amelyek növekvő szénatomszámú alkil-csoportokkal rendelkeznek, eltérő pozíciókban. A *para*-szulfonáto-kalix[n]aréneket hatóanyag-komplexáló tulajdonságuk, valamint sokoldalú biológiai aktivitásuk miatt széles körben tanulmányozták már, azonban a paracelluláris anyagtranszportra gyakorolt hatásuk ezidáig még nem volt ismert.

A sejtéletképességi és hemolízis vizsgálatok hozzásegítettek az egyes α-CD-ek rangsorolásához, továbbá a vegyületek különböző rendszerekben mért toxikussága is összevethetővé vált. A megegyező kémiai módosításokon átesett α- és β-CD-ek biokompatibilitása rávilágított a CD-gyűrű mértének jelentőségére. Egyértelmű összefüggést fedeztünk fel a toxicitás és a szabad hidroxil-csoportok száma között. A hosszú alkil-csoporttal rendelkező CD-ek rossz oldékonysága további kémiai módosításokat tett szükségszerűvé; a szulfát csoportok jelenléte jótékony hatással volt az oldhatóságra, és a citotoxicitásra is. A szulfatálás a kalixarének oldékonyságát is növelte. A C4S és C8S vegyületek növelték a paracelluláris felszívódás mértékét szubtoxikus koncentrációban, azonban a C6S nem mutatott hasonló hatást. Ezen eredmények további kérdéseket vetnek fel a pontos hatásmechanizmusról.

Eredményeink rávilágítanak a makrociklusok szerkezetének és biokompatibilitásának összefüggéseire, valamint ezen ismeretek fontosságára annak érdekében, hogy minden formulációban a legbiztonságosabb segédanyagok legyenek alkalmazhatóak.

Résumé

La faible solubilité de certains médicaments cause des problèmes majeurs dans les formulations pharmaceutiques, puisque la solubilité dans l'eau est un critère indispensable pour la biodisponibilité. Les composés macrocycliques tels que les CDs et les calixarènes ont une cavité relativement hydrophobe, leur permettant ainsi d'encapsuler de nombreuses molécules. Les CDs ont déjà été utilisées comme excipients pharmaceutiques pour l'amélioration de la solubilité. La structure de ces macrocycles permet d'effectuer de nombreuses modifications, qui causent des changements tant au niveau de leurs caractéristiques physico-chimiques que sur leurs effets sur les organismes vivants. Ainsi, l'évaluation de la biocompatibilité de ces dérivés est primordiale en vue de leur utilisation en pharmacie. Puisque l'étude de la biocompatibilité de plusieurs dérivés de β-CD a déjà été étudiée, l'objectif de cette recherche était d'étendre ces expériences à des dérivés de l'α-CD qui sont disponibles dans le commerce. Nous nous sommes intéressés aux relations entre structure et toxicité. Ainsi les dérivés alkyl éther d'α-CD, avec des chaînes alkyle de longueur croissante et substitués sur différentes positions, ont été synthétisés et leur toxicité étudiée.

Les *para*-sulphonato-calix[n]-arènes quant à eux, ont souvent été étudiés et ont montré une forte capacité à complexer de nombreux médicaments. Ils ont aussi démontré une activité biologique polyvalente. Néanmoins, leurs effets sur le mécanisme de transport paracellulaire n'a jamais été évaluée.

Les tests de viabilité cellulaire et d'hémolyse nous ont permis d'une part de classer les α-CDs et de choisir les dérivés les plus sûrs, et d'autre part de comparer leur effets toxiques dans des systèmes différents. La comparaison des α- et ß-CDs portant les mêmes modifications chimiques nous a montré l'importance du nombre d'unités de construction. Le rapport entre l'effet cytotoxique et le nombre de groupes hydroxyles libres est également très important. Les dérivés portant de longues chaînes alkyles possèdent une faible solubilité, ce qui nous a conduits vers d'autres modifications chimiques : la sulfonation de ces derniers dérivés semble avoir un impact bénéfique sur la biocompatibilité de CDs. Elle a aussi amélioré la solubilité des calixarènes. Les calix[4] et [8]arène sulphonates ont prouvé leur effet positif sur l'absorption paracellulaire, tandis que le calix[6]arène sulphonate n'a pas eu d'effet similaire.

Notre recherche conclut que les changements structurels sur les anneaux macrocycliques peuvent avoir un impact majeur sur la biocompatibilité. Comme les possibilités de modification sont pratiquement illimitées, l'évaluation de la structure et de l'activité est indispensable pour faciliter les choix les plus sûrs dans les applications pharmaceutiques à venir.

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XIV. References underlying the dissertation

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List of publications related to the dissertation

1. **Róka, E.**, Ujhelyi, Z., Deli, M. A., Bocsik, A., Fenyvesi, É., Szente, L., Fenyvesi, F., Vecsernyés, M., Váradi, J., Fehér, P., Gesztelyi, R., Félix, C., Perret, F., Bácskay, I.: Evaluation of the Cytotoxicity of [alfa]-Cyclodextrin Derivatives on the Caco-2 Cell Line and Human Erythrocytes.
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IF: 2.465

2. **Róka, E.**, Vecsernyés, M., Bácskay, I., Félix, C., Rhimi, M., Coleman, A. W., Perret, F.: para-Sulphonato-calix[n]arenes as selective activators for the passage of molecules across the Caco-2 model intestinal membrane.
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IF: 6.567

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List of other publications

3. Fehér, P., Ujhelyi, Z., Váradi, J., Fenyvesi, F., **Róka, E.**, Juhász, B., Varga, B., Bombicz, M., Priksz, D., Bácskay, I., Vecsernyés, M.: Efficacy of Pre- and Post-Treatment by Topical Formulations Containing Dissolved and Suspended Silybum marianum against UVB-Induced Oxidative Stress in Guinea Pig and on HaCaT Keratinocytes.
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   IF: 1.003

Total IF of journals (all publications): 18,959
Total IF of journals (publications related to the dissertation): 9,032

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

28 October, 2016

Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

Keywords

α-cyclodextrin, calixarene, macrocycle, biocompatibility, bioavailability, solubility, cytotoxicity, hemolysis, synthesis, MTT, RT-CES, transport

Kulcsszavak

α-ciklodextrin, kalixarén, makrociklus, biokompatibilitás, biohasznosulás, oldékonyság, citotoxicitás, hemolízis, szintézis, MTT, RT-CES, transzport

Mots clés

α-cyclodextrine, calixarène, macrocycle, biocompatibilité, biodisponibilité, solubilité, cytotoxicité, hémolyse, synthèse, MTT, RT-CES, transport

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisors, Dr. Ildikó Bácskay and Dr. Florent Perret. Their continuous support, guidance, patience, motivation, availability and knowledge have helped me along my research work and writing period.

I would like to thank to Professor Romána Zelkó, Dr. Corinne Fruit, Professor Marc Le Borgne and to the members of the jury for their useful comments and advices which intended me to ameliorate my dissertation.

My sincere thanks go to Dr. Miklós Vecsernyés, Professor Julien Leclaire and Professor Hélène Parrot-Lopez, who provided me opportunity to join their research team and who gave me access to their laboratories and research facilities.

I would like to thank to Professor Árpád Tósaki, Professor Stéphane Daniele and Dr. Caroline Félix for providing me the opportunity of fulfilling the co-supervised doctoral research in both universities.

My special thanks go to Professor Lajos Szente, Dr. Éva Fenyvesi and the Cyclolab team who have taught me the most about cyclodextrins, who provided me materials and who answered my every upcoming question with unique professionalism.

I am grateful for the professional help of Professor Anthony W. Coleman, Dr. Mária Deli, Dr. Alexandra Bocsik, Dr. Anikó Borbás, Dr. Erika Mező, Dr. Rudolf Gesztelyi, Dr. Balázs Varga, Dr. Béla Juhász, Mária Horányiné Körei and Katalin Szabó.

I am thankful to Mr. Bob Kaba Loemba, who have supported my progression in any possible way in the frameworks of the French Institute of Hungary.

I am truly thankful to the postdoctoral fellows, PhD and Master students of the CSAp group who helped me not to get lost along my way of chemical synthesis, namely: Geoffroy Germain, Emeric Jeamet, Guillaume Poisson, Yoann Pascal and Mélissa Dumartin.

I am grateful to every colleague of the Department of Pharmaceutical Technology and the CSAp group for all their help and for creating a familial atmosphere in the laboratories.

I am honoured to be the part of the 4th European Conference on Cyclodextrins and the III. International Summer School on Cyclodextrins, where I had been introduced to the infinite possibilities of cyclodextrins.

Last but not least, I have the greatest gratitude towards my Father, my Mother and my Sister who have supported me all along my way, equally in the difficult and joyful periods, from the very beginning. I am especially grateful to Csete Katona for the constant support and stylistic corrections.

This research was realized in the framework of the co-supervised doctoral program of Campus France, organized by the French Institute of Hungary. The project was also supported by the TÉT-14-FR-2015-0031-Balaton project, the GINOP-2.3.2-15 project, and GINOP-2.3.3-15-2016-00021 project (Improvement of research infrastructure: internationalization and network development).

Annexes