Carbohydrate Research 345 (2010) 191–198

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Synthesis, characterization and potential application of monoacyl-cyclodextrins

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ARTICLE INFO

Article history:
Received 10 August 2009
Received in revised form 3 November 2009
Accepted 5 November 2009
Available online 11 November 2009

Keywords:
Cyclodextrin
Regioselectivity
Monoacylation
NMR study
Silibinin

ABSTRACT

Although the preparation of cyclodextrin (CD) monoesters with a variety of carboxylic acids has been already described in the literature, the direct regioselective CD acylation has proved to be critical, often requiring to be replaced with a more elaborate synthetic process. In this paper we describe the one-step preparation of several monoacylated CDs from acyclic or aromatic carboxylic acid derivatives. The ability of β-CD to enclose cupric ions in a sandwich-type manner was exploited to lead to high regioselectivity in the acylation of β-CD with benzoyl chloride, cinnamoyl chloride and phenyl acetyl chloride in water. Long chain aliphatic monoesters of α-, β- and γ-CD were best prepared in DMF. The results of our study showed that solvent and general conditions determined an overwhelming regioselectivity of acylation. ^{1}H, ^{13}C and 2D NMR experiments could easily discriminate the position of the ester. Monoacylated CDs were evaluated as a carrier of silibinin, the inclusion complexes were prepared and characterized by thermal analysis.

1. Introduction

Cyclodextrins (CD) are seductive molecules transcending traditional barriers that separate many subdisciplines of chemical science. They are considered the most important host compounds because of the well-documented ability of the parent CDs to form inclusion complexes with a wide variety of guest species in an aqueous solution.[1] For this reason, CD derivatives are employed in many fields:[2] pharmaceuticals, agrochemicals, analytical chemistry, cosmetics and toiletry, as well as in food industries.[3]

Native β-CD is known to be parenterally nephrotoxic because it removes some cholesterol from the cell walls and transport it to the kidneys where the changes in the 'solution composition' results in the precipitation of the cholesterol/CD complex (pin-like crystals). This cholesterol removal is a common property of cyclodextrin derivatives, in various extents, and it is true particularly for some β-CD derivatives such as methylated β-CD.[4,5] With the aim to mitigate these drawbacks, new CD derivatives are in the offing. The introduction of suitable functional groups on CD hydroxyls may prevent crystallization because it gives rise to amorphous products, and increases the solubility by breaking the 2-OH/3-OH hydrogen bond. The CD regioselective mono- or poly-functionalization in position 2, 3 or 6 still remains a challenging mission. The hydroxyl groups in the three different positions compete for the reagents and make selective derivatization a daunting task that often involves elaborate multistep protection/deprotection strategy.

The regioselective acylation of CDs represents a versatile but difficult means of obtaining series of new derivatives. The derivatization of the primary –OH group (the most basic and nucleophilic), can be driven with a stoichiometric amount of acyl chloride in pyridine, or to avoid the use of such an unpleasant solvent, by displacement of the tosyl group from the 6^{I}-deoxy-6^{I}-(*p*-tolylsulfonyl)-β-CD with a carboxylate.[6,7]

Only very few examples are known of monoacylation at the secondary face of CDs. By the addition of the acyl chloride in acetonitrile to the CD aqueous alkaline solution under precise pH control, the monoacylation occurred mainly on the secondary face and both 2- and 3-monoacyl derivatives were isolated.[8] Recently a monobenzoylation of β-CD by using *N*-benzoylimidazole and carbonate buffer in DMF was described, the authors observed an acyl migration between the C-2 and C-3 hydroxyl groups.[9] Lin and coworkers reported the regioselective C-2 acylation by enzyme-catalyzed transesterification of the vinyl esters.[10,11] In all those cases, the monoacylated derivatives were obtained after time-consuming purifications.

On the basis of the literature data and our previous experience we experimented with several reaction conditions and solvents for the synthesis of regioselective monoacylated CD derivatives. This paper reports the most promising results and the bidimensional NMR spectra to assign the position of the ester unambiguously.

We recently showed that triglyceride-based solid lipid nanoparticles (SLNs) can be prepared in the presence of monoacyl α- and γ-CD derivatives.[12] Along this research line, in this paper, a new

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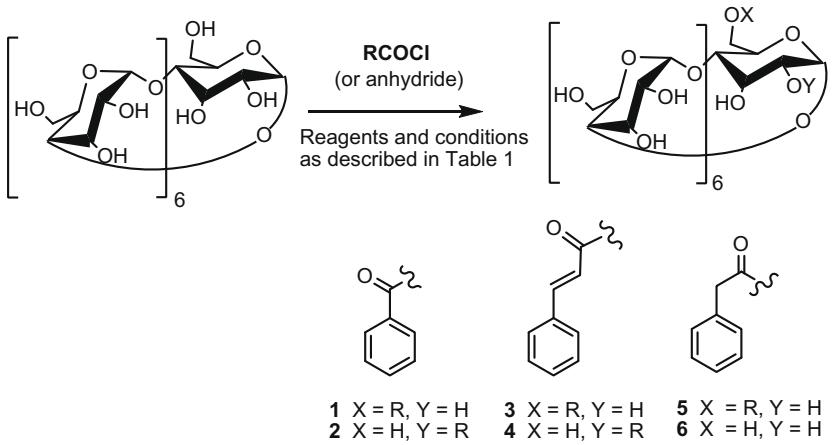
doi:10.1016/j.carres.2009.11.009



Scheme 1. Synthesis of monoacylated β-CD **1**–**6**.

series of monoacylated CD derivatives were preliminarily tested for their ability to form inclusion complexes with silibinin (S), chosen as model drug, in the perspective of a future SLN development.

S is the major active component of silymarin, a mixture of flavolignans extracted from the milk thistle *Silybum marianum*, which presents potent antioxidant activity. Because of the pervasive role that oxidative/nitrosative stress is now known to play in pathophysiology, the flavolignans in silymarin have received increasing attention in treatment and prevention of a variety of liver disorders[13] and in various forms of neoplasies, such as breast, prostate and skin cancer.[14,15] In the literature, the preparation of inclusion complexes of S[16] and other flavonoids such as dioclein[17] and baicalin[18] with β-CD was described, to enhance their low bioavailability after oral administration, related to their low water solubility.

2. Results and discussion

Hydrophobic monoacyl-CDs can be employed as a novel slow-release drug carrier because of their ability to form nanoparticles, vesicles or more elaborate SLN to be proposed for cosmetic or pharmaceutical formulations. Encouraging results on the incorporation of curcumin in SLN by CD inclusion,[12] prompted us to further investigate the improved synthetic protocols for the preparation of CD monoesters. A comprehensive scenario of solvents, catalysts and experimental conditions was explored with the aim to optimize the synthetic protocols of the three common monoacyl derivatives, namely the benzoyl (Bz), cinnamoyl (Cin) and phenylacetyl (PhAc) (Scheme 1). The formation of inclusion complexes with solvents or reagents may strongly perturb the regioselectivity, competing with nucleophilicity and basicity of the three types of hydroxyl groups. As shown in Table 1, the major role of the solvent in the conversion rate and selectivity emerged. The benzoylation in pyridine with a stoichiometric amount of BzCl gave selectively the mono(6-*O*-benzoyl)-β-CD, while the main product in DMF was the mono(2-*O*-benzoyl)-β-CD. The reactivity in DMF was confirmed with the mixed carbonic acid anhydride prepared from ethyl chloroformate. In fact, both the reactions in DMF gave mainly the derivative acylated on the secondary face at the C-2 besides only 2% of mono(6-*O*-benzoyl)-β-CD and traces of diacylated-β-CD. The same reaction repeated in water with Bz mixed anhydride, BzCl or Bz imidazole failed in all cases. When BzCl was added to an aqueous NaOH solution of β-CD and $CuSO_4$ the reaction gave the mono(6-*O*-benzoyl)-β-CD in a 30% yield. The product was isolated by precipitation without the need for purification by chromatography or by soxhlet extraction. In general a slow dropwise addition of the corresponding acyl chloride or anhydride to the CD solution favours the monosubstitution.

As reported in the literature, in an alkaline aqueous solution, β-CD forms a stable copper-CD assembly,[19,20] in which the two CD tori are joined by copper(II) ions in a sandwich-type complex.[21] In this metal-bridged complex the CD secondary face is masked so that new regioselective synthetic protocols can be envisaged,

Table 1
Assessment of esterification on β-CD

Entry	Reagent	Reaction condition	Product (yield %)
1	BzCl	5 h, rt, Pyridine	**1** (8%)[a]
2	Bz anhydride[b]	DMAP, 2 h, rt, DMF	**1** (3%), **2** (14.5%)
3	BzCl	DMAP, 2 h, rt, DMF	**1** (7%), **2** (13%)
4	Bz anhydride[b]	NaOH, 3 h, rt, water	—[c]
5	Bz imidazole	NaOH, 8 h, rt, water	—[c]
6	BzCl	NaOH, 8 h, rt, water	—[c]
7	BzCl	$CuSO_4$, NaOH, 4 h, rt, water, CH_3CN	**1** (30%)
8	Bz anhydride[b]	$CuSO_4$, NaOH, 5 h, rt, water, CH_3CN	—[c]
9	CinCl	DMAP, 2 h, rt, DMF	**4** (15%), **3** (3%)
10	CinCl	$CuSO_4$, NaOH, 4 h, rt, water, CH_3CN	**3** (31%)
11	Cin anhydride[b]	DMAP, 2 h, rt, DMF	**4** (20%), **3** (1%)
12	PhAcCl	DMAP, 2 h, rt, DMF	**5** (15%), **6** (5%)
13	PhAc anhydride[b]	DMAP, 2 h, rt, DMF	**5** (17%), **6** (2%)
14	PhAcCl	$CuSO_4$, NaOH, 4 h, rt, water, CH_3CN	**6** (29%)

[a] Yield as referred by Tong et al.[6]
[b] Mixed carbonic acid anhydride prepared from the corresponding acid with ethyl chloroformate.
[c] Starting material.

Table 2
Assessment of esterification on β-CD

Entry	CD	Reagent	Reaction condition	Product (yield %)
1	β	Propanoyl Cl	$CuSO_4$, water, CH_3CN, 5 h, rt	—[a]
2	β	Ethylmalonyl Cl	$CuSO_4$, water, CH_3CN, 5 h, rt	—[a]
3	β	Isostearoyl Cl	$CuSO_4$, water, CH_3CN, 5 h, rt	—[a]
4	β	Isostearic anhydride[b]	DMF, 2 h, rt	**7** (16), **8** (5)
5	β	Isostearoyl Cl	DMAP, Et_3N, DMF, 2 h, rt	**7** (25), **8** (10)
6	α	Isostearoyl Cl	DMAP, Et_3N, DMF, 2 h, rt	**9** (40), **10** (13)
7	γ	Isostearoyl Cl	DMAP, Et_3N, DMF, 2 h, rt	**11** (16), **12** (7)

[a] Starting material.
[b] Mixed carbonic acid anhydride prepared from the corresponding acid with ethyl chloroformate.

as referred to the synthesis of the mono(6-*p*-toluensulfonyl)-β-CD.[22]

The acylation protocol was optimized by the addition of 8 equiv of BzCl dissolved in CH_3CN to a basic aqueous solution of $CuSO_4$ and β-CD. The monoacyl derivative was isolated by precipitation after addition of 1 N HCl sol. to neutralize the reaction mixture and NMR experiments confirmed the ester moiety on the primary face. The same reaction conditions were used with cinnamoyl chloride and phenylacetyl chloride (DMF vs water). In these two cases, DMF disfavours the attack on the primary face while the mono-(6-acyl)-β-CD derivative was obtained when the reaction was performed in water in the presence of $CuSO_4$. Surprisingly, with phenylacetyl chloride the regiochemistry was inverted: mono-(6-*O*-phenylacetyl)-β-CD (**5**) was rather selectively obtained in DMF while mono(2-*O*-phenylacetyl)-β-CD (**6**) was obtained in water, only traces of the C-6 esterified derivative were detected. Few studies in the literature describe the binding constants of β-CD with benzoic acid and phenylacetic acid, so the existence of intracavity inclusion of these derivatives has been proved.[23,24] Braga et al. published the characterization of the crystal structure of the inclusion complex isolated in water from β-CD with *S*-(+)-ibuprofen (a phenylacetic derivative).[25] By the combination of powder X-ray diffraction (XRD) and single crystal X-ray diffraction, they confirmed the formation of a head-to-head dimer of β-CD molecules. The formation of a phenylacetyl-CD assembly that masks the primary face, clearly unables the attack on the C-6 position. These data explain the inverse 2→6 regioselectivity observed in water, respectively with phenylacetyl chloride and benzoyl chloride.

To broaden the scope, we extended our study to the esterification of CD with some aliphatic carboxylic acids; a few examples are listed in Table 2, which includes comparative data for different alkyl acylating agents. Esterification with propanoyl, ethylmalonyl and isostearyl chloride in water with $CuSO_4$, failed. When the reaction was performed in DMF, the isostearoyl-mixed carbonic acid anhydride prepared with ethyl chloroformate gave mainly the mono(6-*O*-isostearoyl derivative)-β-CD (**7**) in addition to the regioisomer **8** and traces of disubstituted derivatives (Table 2, entry 4). Higher conversion was observed when the reaction was repeated in DMF in the presence of isostearoyl chloride (Table 2, entry 5). Scheme 2 reports all the monoisostearoyl derivatives obtained under these conditions.

With the aim to obtain new hydrophobic CD derivatives and to compare their ability to form an inclusion complex with silibinin (2*R*,3*R*)-2-[(2*R*,3*R*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one) (Fig. 1), mono (6-*O*-isostearoyl derivative)-α and γ-CD were also synthesized. As described for the β-CD, also the α- and γ-CD can be esterified in DMF affording the ester on the primary face as the main product. All the products were characterized by mass spectrometry and NMR spectroscopy. NMR spectra enable the unambiguous determination of the regiochemistry as shown in Table 2 and in the experimental part.

As reported in the well-known NMR study of Yoshimoto,[26] the shift of *O*-acylated carbon ($^*CH_2OCOR$) is somewhat downfielded while the adjacent carbon C_β ($^*CCH_2OCOR$) would move markedly upfield in ^{13}C NMR. Furthermore, throughout all positional isomers, the anomeric proton signal in ^1H NMR of the acylated glucose unit resonates downfield with a characteristic doublet coupling pattern.

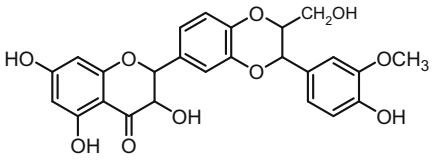
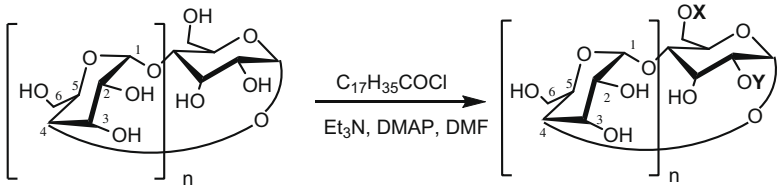
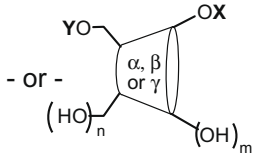


Figure 1. Chemical structure of silibinin.



7 n = 6, m = 13, **X** = $COC_{17}H_{35}$, **Y** = H
8 n = 6, m = 13, **X** = H, **Y** = $COC_{17}H_{35}$
9 n = 5, m = 11, **X** = $COC_{17}H_{35}$, **Y** = H
10 n = 5, m = 11, **X** = H, **Y** = $COC_{17}H_{35}$
11 n = 7, m = 15, **X** = $COC_{17}H_{35}$, **Y** = H
12 n = 7, m = 15 , **X** = H, **Y** = $COC_{17}H_{35}$



Scheme 2. Synthesis of monoisostearoyl-α-, β-, γ-CD **7**–**12**.

Table 3
Chemical shift of ^{13}C NMR of monosubstituted derivatives **1**, **3**, **6**

Carbon atom	**1** DMSO-d_6	**3** DMSO-d_6	**6** DMSO-d_6
C-1	102.65	103.02	102.41
	102.02	102.97	102.21
	101.61		102.14
C-1′			98.87
C-2	72.44	73.26	72.71
	72.25	73.46	72.32
			72.25
C-2′			73.55
C-3	73.1	73.89	73.66
	73.09		73.47
C-3′			70.26
C-4	82.58	83.14	81.39
	82.44	82.72	81.27
	81.29	82.19	81.10
		82.00	80.70
C-5	72.28	72.91	72.54
	72.13	72.10	72.44
	72.01	72.02	
C-5′	69.07	69.75	
C-6	59.97	60.77	60.28
	59.68		
C-6′	64.06	64.47	
C=O	165.69	166.97	174.18
	133.47 (C_{ipso})	145.42 (Ph–CH=	40.76 (CH_2–Ph)
	129.85 (C_{para})	134.93 (C_{ipso})	134.32 (C_{ipso})
	128.82 C_{ortho})	129.22 (C_{para})	129.82 (C_{ortho})
	129.55 (C_{meta})	129.87 (C_{ortho})	128.15(C_{meta})
		129.25 (C_{meta})	126.76(C_{para})
		118.86 (CH–CO)	

As an example, analysis of the ^{13}C NMR spectra of compounds **1**, **3** and **5** revealed that an esterification occurred at the C-6 primary hydroxyl position, while compounds **2**, **4** and **6** referred to the acylated in C-2. In Table 3, the chemical shifts of ^{13}C NMR of β-CD derivatives **1**, **3** and **6** obtained by performing the reaction in water are listed. In the ^{13}C NMR spectrum of products **1** and **3** a peak at 64.06 or 64.24 ppm referred to the downshift of the C-6′ substituted carbon, at 69.07 and 69.51 ppm the NMR spectra evidenced the upshift of the C-5′ adjacent carbon. On the contrary, the mono(2-*O*-phenylacetyl)-β-CD (**6**) showed the downfield shift of the C-2′ signal at 73.55 ppm while the two upfield peaks referred to the C-1′ at 98.87 and to the C-3′ at 70.26 ppm. As described previously, even the ^1H NMR spectra provided substantial information. The mono(6-*O*-acyl)-β-CD (**1**) and **3** showed a downfield signal coming from the proton linked to the C-6′ atom, detected by homo- and hetero-nuclear 2D NMR experiments and overlaid by the OH(6) signal. The ^1H NMR spectrum of product **6** showed a C-1′ proton downfield doublet (δ = 5.25 ppm) separate from the other six C-1 protons (multiplet δ = 5.06 ppm), even the C-3 protons show a downfield triplet at 4.14 ppm.

A preliminary evaluation on the ability of acyl-β-CD to include S was performed by characterizing the S-CD inclusion complexes of a set of different acyl-CDs with differential scanning calorimetry (DSC). The S transition peak was monitored: DSC analysis was used to ascertain the formation of inclusion complexes and to compare them with the plain physical mixtures. The thermograms reported in Figures 2A and B showed the formation of S complexes with all CD-derivatives tested.

In the isostearoyl CD series (Fig. 2A), the S transition peak (158.37 °C) disappeared completely in 2:1 CD:S molar ratio inclusion complexes prepared with each CD, probably indicating a complete inclusion of the drug in the CD cavity. On the other hand, with γ-CD a complete disappearance of the transition peak was already observed at 1:1 CD:S molar ratio. Probably the bigger size of the γ-CD should favour the inclusion of S molecule. In the S-CD derivatives physical mixture, the S transition peak was still present, and no significant modification was noted, indicating only slight interactions.

As the complete ethanol removal at room temperature by N_2 stream can be questioned, and considering that the presence of ethanol has been described to influence the complexation of drugs with CD,[27] the complex prepared by dissolving S and α-isostearoyl-CD (1:2 molar ratio) in acetone was analyzed by DSC. The obtained curve is quite superimposable to that obtained in ethanol.

In the β-CD series (Fig. 2B), a complete disappearance of the S transition peak was observed in all S/CD derivatives 1:2 molar ratio complexes, confirming the results obtained with isostearoyl β-CD.

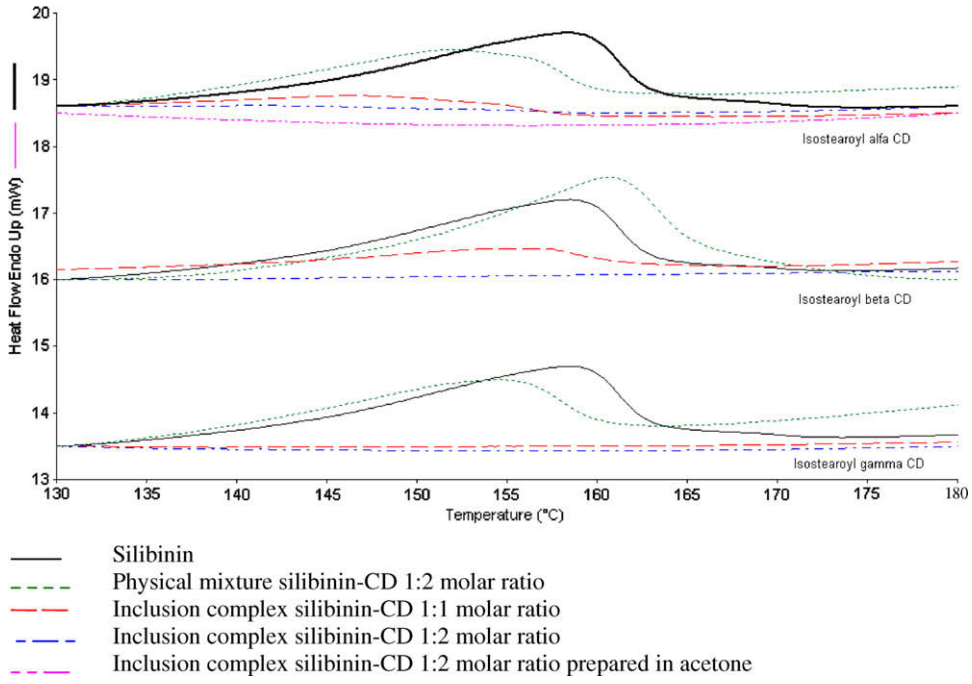


Figure 2A. DSC thermograms of *S*-isostearoyl (α, β, γ)-CD **7**, **9** and **11**.

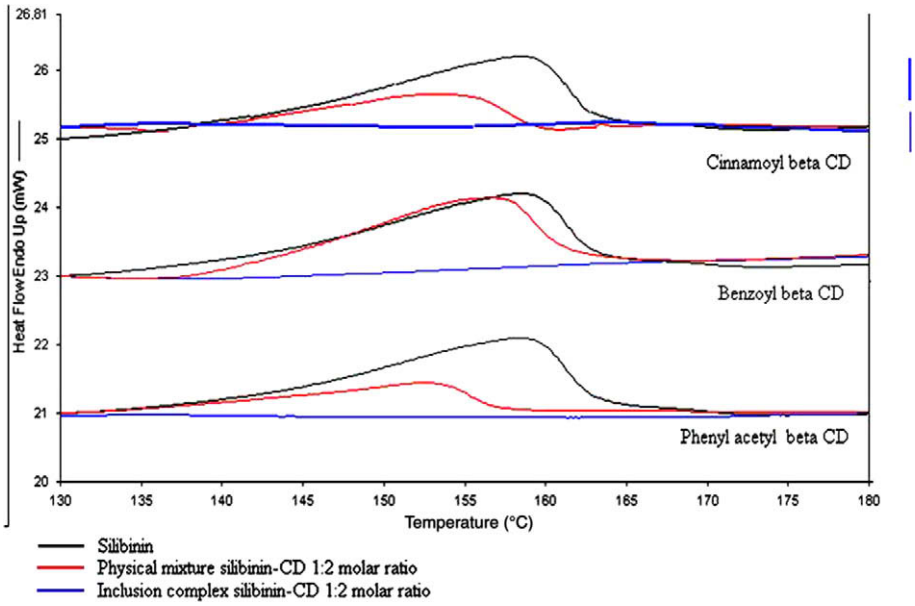


Figure 2B. DSC thermograms of *S*-acyloyl β-CD **1**, **3** and 6.

To confirm the S/isostearoyl γ-CD 1:1 inclusion complex formation, the stoichiometry of the complex was evaluated by the continuous variation method (Job's plot).[28] The *r* ratio was plotted against the difference of absorbance (ΔA) of drug in the presence and in the absence of isostearoyl γ-CD. The results, reported in Figure 3 evidenced a maximum ΔA at *r* value 0.5, indicating a 1:1 stoichiometry.

As also reported in the literature,[29] the DSC curves of the raw material compared with those obtained by simply mixing acyl-CDs and S, and with those of inclusion complexes, confirm not only an interaction between flavonoid and acyl-CDs, but also a real inclusion.

3. Conclusions

In summary, the direct synthesis of a series of monoacylated CDs in water and DMF has been described. From this study, it emerged that a general procedure for the direct regioselective CD monoacylation is not available. On the basis of CD type and acylating agent, ad hoc tailored methods have to be applied to achieve regioselective monoacylation. Good regioselectivity was observed when the reactions were performed DMF by using mixed anhydrides or in water/$CuSO_4$ with aromatic acyl chlorides. The intriguing influence of the solvent often showed complementary regioselectivity. The ability to include S was successfully evaluated by performing DSC on the S-complex with mono(6-Bz)-β-CD, mono(6-Cin)-β-CD and mono(2-PheAc)-β-CD, and mono(6-isostearoyl)α-, β- and γ-CD.

Such promising results will require further investigations for a future development of SLN-containing S/CD complexes in drug delivery.

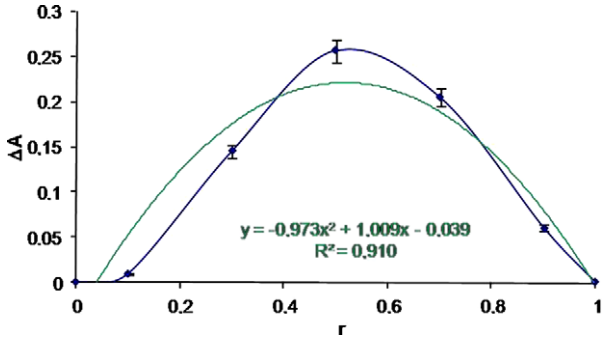


Figure 3. Continuous variation plot of *S*-monoisostearoyl γ-CD in 1.0% DMSO aqueous solution.

4. Experimental

Commercially available reagents and solvents were used without further purification. Native CDs were kindly provided by Wacker Chemie. In accordance with the well-established procedure, the mixed carbonic acid anhydrides were prepared from the corresponding acid with ethyl chloroformate in dichlorometane. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or by heating after a spray with 5% H_2SO_4 in ethanol. IR spectra were recorded with a Shimadzu FT-IR 8001 spectrophotometer. NMR spectra were recorded with a Bruker 300 Avance (300 MHz and 75 MHz for ^1H and ^{13}C, respectively) at 25 °C; chemical shifts are calibrated to the residual proton and carbon resonances of the solvent: DMSO-d_6 ($\delta H = 2.54$, $\delta C = 39.5$). Chemical shifts (δ) are given in ppm, coupling constants (J) in hertz. ESI-mass spectra were performed on a Waters Micromass ZQ spectrometer equipped with ESI source. A DSC7 Perkin Elmer was used for differential scanning calorimetry measurements.

4.1. Mono(6-*O*-benzoyl)-β-CD (1)

β-CD (1 g, 0.88 mmol) was dissolved in distilled water (25 ml) at 60 °C. A copper sulfate solution (0.66 g, 2.65 mmol) in water (15 ml) was added. The reaction mixture was stirred at room temperature for 2 h then a NaOH solution (0.85 g, 21.25 mmol) was added. A benzoyl chloride (0.8 ml, 6.95 mmol) solution in CH_3CN (7.5 ml) was added dropwise and stirred for 4 h at room temperature. During the addition of benzoyl chloride the solution became opalescent. After 4 h, 1 N HCl was added to neutralize the reaction mixture. Precipitate was observed after the addition of HCl and it was filtered. The precipitate was dissolved in methanol and the desired monoacyl derivative was precipitated using acetone (3 volumes). Further recrystallization using hot methanol yielded

367 mg (33% yield) of pure product. Analytical data were in accordance with published data.[6]

4.2. Mono(2-*O*-benzoyl)-β-CD (2)

β-CD (0.2 g, 0.176 mmol) and ethylbenzoylcarbonate (51 mg, 0.264 mmol) were dissolved in 4 ml of DMF. A catalytic amount of 4-dimethylaminopyridine (DMAP) was added. The solution was left under stirring for 2 h at room temperature. The solution was partially dried under vacuum therefore acetone was added and the precipitate was filtered. The desired product was isolated by reverse-phase chromatography with a gradient from water/methanol 97:3 to methanol 100%.

31 mg (14.5% yield) of mono-(2-*O*-benzoyl) β-CD were recovered and characterized. Analytical data were in accordance with reported values.[8]

4.3. Mono(6-*O*-cinnamoyl)-β-CD (3)

β-CD (1 g, 0.88 mmol) was dissolved in distilled water (45 ml) at 60 °C. A copper sulfate solution (0.66 g, 2.65 mmol) in water (65 ml) was added. The reaction mixture was stirred at room temperature for 2 h then a NaOH solution (0.88 g, 21.25 mmol) in water (25 ml) was added. A solution of *t*-cinnamoyl chloride (0.8 ml, 7 mmol) in CH_3CN (7.5 ml) was added dropwise and stirred for 4 h at room temperature. After 4 h, 1 N HCl was added to neutralize the reaction mixture. Precipitate was observed after the addition of HCl, so that the reaction mixture was filtered to remove copper salt. To the filtrate acetone was added to precipitate β-CD. The resultant filtrate was evaporated and the desired product was precipitated in water to yield 337 mg (31% yield) of the desired cinnamoyl derivative.

Compound 3 is a white powder; R_f = 0.4 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2925, 1736, 1653, 1471, 1369, 1086, 1028 and 754; ^1H NMR (300 MHz; DMSO-d_6): 7.69 (m, 3H, *ortho*, –C*H*–CO), 7.46 (m, 3H, *meta, para*), 6.72 (d, 1H, *J* = 15.7 Hz, Ph–C*H*═), 5.76 (m, 14H, O(2)H, O(3)H), 4.86 (br s, 7H, H-1), 4.54–4.43 (m, 7H, O(6)H, H-6$^{A'}$), 4.22 (m, 1H, H-6$^{B'}$), 3.96 (m, 1H, H-5′), 3.7–3.2 (m, overlaps with H_2O, H-6, H-3, H-2, H-4, H-5); ^{13}C NMR (75 MHz; DMSO-d_6): as shown in Table 3, *m/z* (ESI) calcd for $C_{51}H_{76}O_{36}$ 1287.40 $[M+Na]^+$, found 1287.75 $[M+Na]^+$.

4.4. Mono(2-*O*-cinnamoyl)-β-CD (4)

β-CD (0.5 g, 0.44 mmol) and *t*-ethylcinnamoylcarbonate (145 mg, 0.65 mmol) were dissolved in 10 ml of DMF. A catalytic amount of DMAP was added. The solution was left under stirring for 2 h at room temperature. The solution was partially dried under vacuum therefore acetone was added and the precipitate was filtered. The desired product was isolated by reverse-phase chromatography with a gradient from water/methanol 97:3 to methanol 100%.

Hundred and eighteen milligrams (20% yield) of mono-(2-*O*-cinnamoyl)-β-CD were recovered and characterized.

Compound **4** is a light yellow powder; R_f = 0.5 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2925, 1736, 1653, 1471, 1369, 1086, 1028 and 754; ^1H NMR (300 MHz; DMSO-d_6): 7.69 (m, 3H, *ortho*), 7.58 (d, 1H, *J* = 15.9 Hz, CH–CO), 7.43 (m, 3H, *meta, para*), 6.57 (d, 1H, *J* = 15.9 Hz, Ph–C*H*═), 5.74 (m, 13H, O(2)H, O(3)H), 5.08 (m, 1H, H-1′), 4.75 (br s, 6H, H-1), 4.58–4.24 (m, 7H, O(6)H, H-2′), 3.83 (m, 1H, H-3′), 3.6–3.2 (m, overlaps with H_2O, H-6, H-3, H-2, H-4, H-5); ^{13}C NMR (75 MHz; DMSO-d_6): 167.15 (CO), 143, 7 (Ph–CH═), 135.25 (C_{ipso}), 130.87 (C_{meta}), 129.74 (C_{para}), 129.01 (C_{ortho}), 120.64 (═CH–CO), 102.87 (C1), 100.01 (C1′), 82.44 (C4), 73.93 (C2′), 72.86 (C3), 72.63 (C2), 72.06 (C5), 70.35 (C3′), 60.78 (C6); *m/z* (ESI) calcd for $C_{51}H_{76}O_{36}$ 1287.40 $[M+Na]^+$, found 1287.36 $[M+Na]^+$.

4.5. Mono(6-*O*-phenylacetyl)-β-CD (5)

β-CD (0.2 g, 0.176 mmol) and ethylphenylacetylcarbonate (55 mg, 0.26 mmol) were dissolved in 4 ml of DMF. A catalytic amount of DMAP was added. The solution was left under stirring for 2 h at room temperature. The solution was partially dried under vacuum therefore acetone was added and the precipitate was filtered. The desired product was isolated by reverse-phase chromatography with a gradient from water/methanol 97:3 to methanol 100% to yield 36 mg (17% yield) of the desired mono-(6-*O*-phenylacetyl)-β-CD.

Compound **6** is a white powder; R_f = 0.42 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2924, 1732, 1660, 1475, 1372, 1089, 1028 and 754; ^1H NMR (300 MHz; DMSO-d_6): 7.30 (m, 5H, aromatic protons), 5.84–5.73 (m, 14H, O(2)H, O(3)H), 4.86 (br s, 7H, H-1), 4.76–4.68 (m, 6H, O(6)H), 4.46 (m,2H, CH_2Ph), 4.38 (m, 1H, H-6$^{A'}$), 4.12 (m, 1H, H-6$^{B'}$) 3.9–3.2 (m, overlaps with H_2O, H-6, H-3, H-2, H-4, H-5); ^{13}C NMR (75 MHz; DMSO-d_6): 172.15 (CO), 135.2 (C_{ipso}), 130.30 (C_{ortho}), 129.10 (C_{meta}), 127.58 (C_{para}), 102.86 (C1), 82.65 (C4), 82.65 (C4), 73.88(C3), 73.27 (C2), 72.90 (C5), 68.78 (C5′), 63.77 (C6′), 61.12 (C6); *m/z* (ESI) calcd for $C_{50}H_{76}O_{36}$ 1275.41 $[M+Na]^+$, found 1275.61 $[M+Na]^+$.

4.6. Mono(2-*O*-phenylacetyl)-β-CD (6)

β-CD (1 g, 0.88 mmol) was dissolved in distilled water (45 ml) at 60 °C. A copper sulfate solution (0.66 g, 7 mmol) in water (65 ml) was added. The reaction mixture was stirred at room temperature for 2 h then a NaOH solution (0.88 g, 21.25 mmol) in water (25 ml) was added. Then, phenylacetyl chloride (0.9 ml, 6.95 mmol) solution in CH_3CN (7.5 ml) was added dropwise and stirred for 4 h at room temperature. 1 N HCl was added to neutralize the reaction mixture and precipitate was observed. To the filtrate solution, acetone was added to precipitate β-CD. The resultant filtrate was evaporated and the desired product was isolated by reverse-phase chromatography with a gradient from water/methanol 97:3 to methanol 100% to yield 305 mg (29% yield) of the desired mono-(2-*O*-phenylacetyl)-β-CD.

Compound **6** is a light yellow powder; R_f = 0.52 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2925, 1730, 1659, 1475, 1372, 1089, 1028 and 754; ^1H NMR (300 MHz; D_2O): 7.32 (m, 5H, aromatic protons), 5.25 (d, 1H, *J* = 3.6 Hz, H-1′), 5.06–5.01 (m, 6H, H-1), 4.8 (m, overlaps with H_2O,H-2′), 4.16 (t, 1H, *J* = 7.8 Hz, H-3′), 4.0–3.48 (m, 35H, H-6, H-3, H-2, H-4, H-5, CH_2CO); ^{13}C NMR (75 MHz; DMSO-d_6): as shown in Table 3, *m/z* (ESI) calcd for $C_{50}H_{76}O_{36}$ 1275.41 $[M+Na]^+$, found 1275.26 $[M+Na]^+$.

4.7. General procedure for the synthesis of monoisostearoyl-α-, β-, γ-CD derivatives

CD (0.51 mmol), and triethylamine (56 μl, 0.76 mmol) were dissolved in dry DMF (3 ml). A catalytic amount of DMAP was added. The solution was cooled down to −15 °C and the isostearoyl chloride (0.22 g, 0.76 mmol) previously diluted with DMF (2 ml), was added dropwise. The reaction mixture was stirred for 2 h at room temperature, and finally filtered on a sintered glass funnel to remove triethylammonium chloride. The solution was dried under vacuum and the crude product was purified by reverse-phase chromatography (from water/methanol 97:3 to methanol 100%) to give pure compounds **7–12**.

4.7.1. Mono(6-*O*-isostearoyl)-β-CD (7)

White powder (25%); R_f = 0.57 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2926, 1736, 1653, 1369, 1157, 1028; ^1H NMR (300 MHz; DMSO-d_6): 5.78–5.69 (m, 14H, O(2)H, O(3)H), 4.83 (br s, 7H, H-1), 4.47–4.38 (m, 6H, O(6)H), 3.35 (m,

1H, H-6$^{A'}$), 4.073.35 (m, 1H, H-6$^{B'}$), 3.82 3.35 (m, 1H, H-5′), 3.63 (m, 29H, H-3, H-5, H-6), 3.5–3.2 (m, overlaps with H_2O, H-2,H-4), 2.30 (m, 2H, CH_2CO), 1.49 (m, 2H, CH_2), 1.28 (m, 23H, CH_2, CH), 0.89 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 172.12 (CO), 101.94 (C1), 81.53 (C4), 73.08 (C3), 72.45 (C2), 72.054 (C5), 69.05 (C5′), 63.52 (C6′), 59.93 (C6), 31.31 (CH_2CO), 29.04 (CH_2), 28.76 (CH_2), 24.52 (CH_2), 22.12 (CH_2), 15.20 (CH_2), 14.03 (CH_3); *m/z* (ESI) calcd for $C_{60}H_{104}O_{36}$ 1400.63, found 1423.55 $[M+Na]^+$.

4.7.2. Mono(2-*O*-isostearoyl)-β-CD (8)

Light yellow powder (10%); R_f = 0.63 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2926, 1736, 1653, 1369, 1157, 1028; ^1H NMR (300 MHz; DMSO-d_6): 5.68–5.42 (m, 14H, O(2)H, O(3)H), 5.02 (d, 1H, *J* = 3.2 Hz, H-1′), 4.84 (br s, 6H, H-1), 4.54(m, 7H, O(6)H), 4.40 (d, *J* = 3.2 Hz, H-2′), 4.05 (m, 1H, H-3′), 3.8–3.6 (m, 28H, H-3, H-5, H-6), 3.45–3.33 (m, overlaps with H_2O, H-2,H-4), 2.32 (m, 2H, CH_2CO), 1.48 (m, 2H, CH_2), 1.2–1.4 (m, 23H, CH_2, CH), 0.72 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 174.12 (CO), 103.14 (C1), 100.1 (C1′), 82.5 (C4), 73.87 (C3), 73.74 (C2), 73.154 (C5), 69.86 (C3′), 60.1 (C6), 31.27 (CH_2CO), 29.12 (CH_2), 28.56 (CH_2), 23.73 (CH_2), 22.42 (CH_2), 15.47 (CH_2), 14.63 (CH_3) *m/z* (ESI) calcd for $C_{60}H_{104}O_{36}$ 1400.63, found 1423.43 $[M+Na]^+$.

4.7.3. Mono(6-*O*-isostearoyl)-α-CD (9)

White powder (40%); R_f = 0.56 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1}: 2926, 2856, 1736, 1659, 1153, 1080, 949, 704; ^1H NMR (300 MHz; DMSO-d_6): 5.67–5.42 (m, 12H, O(2)H, O(3)H), 4.85 (br s, 6H, H-1), 4.62–4.42 (m, 7H, O(6)H, H-6′), 4.0–3.2 (m, overlaps with H_2O, H-2,H-4, H-3, H-5, H-6), 2.49 (m, 2H, CH_2CO), 1.55 (m, 2H, CH_2), 1.0–1.42 (m, 23H, CH_2, CH), 0.88 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 173.22 (CO), 101.98 (C1), 82.08 (C4), 73.29 (C3), 72.23 (C2), 72.1 (C5), 68.48 (C5′), 62.53 (C6′), 60.01 (C6), 32.21 (CH_2CO), 29.14 (CH_2), 28.57 (CH_2), 24.47 (CH_2), 21.72 (CH_2), 15.20 (CH_2), 13.73 (CH_3) *m/z* (ESI) calcd for $C_{54}H_{94}O_{31}$ 1238.58, found 1262.32 $[M+Na]^+$.

4.7.4. Mono(2-*O*-isostearoyl)-α-CD (10)

Light yellow powder (13%); R_f = 0.63 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2926, 1736, 1653, 1369, 1157, 1028; ^1H NMR (300 MHz; DMSO-d_6): 5.67–5.43 (m, 11H, O(2)H, O(3)H), 5.03 (d, 1H, *J* = 3.2 Hz, H-1′), 4.86 (br s, 5H, H-1), 4.75 (d, 1H, *J* = 3.2 Hz, H-2′), 4.6–4.35 (m, 6H, O(6)H), 4.11–4.05 (m, 1H, H-3′), 4.05–3.23 (m, overlaps with H_2O, H-2,H-4, H-3, H-5, H-6), 2.27 (m, 2H, CH_2CO), 1.57 (m, 2H, CH_2), 1.2–1.3 (m, 23H, CH_2, CH), 0.92 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 173.12 (CO), 103.74 (C1), 98.75 (C1′), 82.22 (C4), 73.18 (C3), 73.22 (C2′), 72.15 (C2), 71.75 (C5), 69.91 (C3′), 60.0 (C6), 31.32 (CH_2CO), 29.42 (CH_2), 28.76 (CH_2), 23.43 (CH_2), 22.52 (CH_2), 15.67 (CH_2), 14.33 (CH_3) *m/z* (ESI) calcd for $C_{54}H_{94}O_{31}$ 1238.58, found 1262.12 $[M+Na]^+$.

4.7.5. Mono(6-*O*-isostearoyl)-γ-CD (11)

White powder (16%); R_f = 0.58 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1}: 2925, 2859, 1738, 1661, 1153, 1080, 949, 704; ^1H NMR (300 MHz; DMSO-d_6): 5.81–5.79 (m, 16H, O(2)H, O(3)H), 4.90 (br s, 8H, H-1), 4.62–4.42 (m, 9H, O(6)H, H-6′), 4.0–3.2 (m, overlaps with H_2O, H-2,H-4, H-3, H-5, H-6), 2.49 (m, 2H, CH_2CO), 1.55 (m, 2H, CH_2), 1.0 -1.42 (m, 22H, CH_2), 0.88 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 175.22 (CO), 101.55 (C1), 83.08 (C4), 75.29 (C3), 72.56 (C2), 71.65 (C5), 69.64 (C5′), 63.54 (C6′), 60.68 (C6), 33.25 (CH_2CO), 28.25 (CH_2), 27.63 (CH_2), 24.20 (CH_2), 20.65 (CH_2), 15.23 (CH_2), 14.76 (CH_3) *m/z* (ESI) calcd for $C_{66}H_{114}O_{41}$ 1562.68, found 1575.32. $[M+Na]^+$.

4.7.6. Mono(2-*O*-isostearoyl)-γ-CD (12)

Light yellow powder (7%); R_f = 0.65 (*i*PrOH/H_2O/AcOEt/NH_4OH 5:3:1:1); ν_{max} (KBr)/cm^{-1} 3420, 2926, 1735, 1655, 1369, 1157, 1028; ^1H NMR (300 MHz; DMSO-d_6): 5.80–5.75 (m, 15H, O(2)H, O(3)H), 5.13 (d, 1H, *J* = 3.2 Hz, H-1′), 4.90 (br s, 7H, H-1), 4.73 (d, *J* = 3.2 Hz, H-2′), 4.62–4.32 (m, 8H, O(6)H), 4.05–4.01 (m, 1H, H-3′), 4.0–3.15 (m, overlaps with H_2O, H-2,H-4, H-3, H-5, H-6), 2.15 (m, 2H, CH_2CO), 1.67 (m, 2H, CH_2), 1.2–1.3 (m, 22H, CH_2), 0.92 (m, 8H, CH_3, CH_2); ^{13}C NMR (75 MHz; DMSO-d_6): 172.12 (CO), 103.65 (C1), 96.75 (C1′), 91.95 (C4), 73.13 (C3), 72.77 (C2′), 72.15 (C2), 71.60 (C5), 69.83 (C3′), 59.30 (C6), 32.15 (CH_2CO), 29.13 (CH_2), 27.82 (CH_2), 24.15 (CH_2), 22.55 (CH_2), 16.23 (CH_3), 14.33 (CH_3) *m/z* (ESI) calcd for $C_{66}H_{114}O_{41}$1562.68, found 1585.73 $[M+Na]^+$.

4.8. Silibinin/CD derivatives inclusion complexes

S/CD-derivatives complexes at 1:1 and 1:2 molar ratios were prepared. Appropriate amounts of CD derivatives and S were dispersed/solubilized in 3 ml of ethanol. The organic solvent was then removed by keeping the suspension under a nitrogen stream for 30 min.

4.9. DSC analysis

An appropriate amount of each sample, corresponding to 0.7 mg S was placed in conventional aluminium pans and a scan speed of 10 °C min^{-1} was used operating in 120–180 °C temperature range. (DSC7 Perkin Elmer).

4.10. Continuous variation method (Job's plot)

Equimolar (0.05 mM) solutions of S and isostearoyl γ-CD in 1.0% V/V DMSO aqueous solution were added in varying quantities so as to get different *r* values: *r* = [S]/([S] + [isostearoyl γ-CD]) at a 2 ml final volume. Samples were analyzed using UV spectrophotometer at 286 nm.

Acknowledgements

Regione Piemonte and the University of Turin (Project Nano-IGT—Converging technologies) are warmly acknowledged for the financial support. D. S. P. is indebted to the University of Turin for the young researcher fellowship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.11.009.

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