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ORIGINAL ARTICLE

Novel chemo-enzymatic access to amphiphilic cyclodextrins

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Abstract A new class of mono-substituted amphiphilic cyclodextrins was synthesized in a one-step lipase catalysed amidification. Microbial and animal lipases were able to catalyse this reaction. Depending on the acyl donors, various hydrophobic moieties have been grafted on methylated β-cyclodextrin. Azoninyl-methylated-β-cyclodextrin derivatives were also obtained by reaction of acetaldehyde and modified cyclodextrins in presence of dedicated lipase.

Keywords Amidification · Amphiphilic · Cyclodextrin · Fatty acids · Lipase · Solvent free medium · Vinyl esters

Introduction

Cyclodextrins are cyclic oligosaccharides composed of 6, 7 or 8 α-D-(1 $\rightarrow$ 4) glucopyranoside moieties. They are recognized to have significant potential as drug carriers arising from inclusion complexes. Amphiphilic derivatives based on cyclodextrins, namely phospholipidylcyclodextrins, were shown as able to go through the blood brain barrier without breaking its integrity [1]. They were prepared from a pure phospholipid (DMPE) onto cyclodextrins through a spacing arm [2, 3]. The methylated derivatives were shown to self-organize in water with low CMC to form fluctuating micellar fibers retaining inclusion capacity of the cyclodextrin cavities.

To overcome the drawbacks encoutered with the Phospholipidyl-CDs synthesis, namely high cost, tedious synthesis and rather poor versatility of the chemical structures and stability, a new class of amphiphilic CDs should be designed based on the chemo-enzymatic ways. Hydrolytic enzymes are able to catalyse efficiently a wide variety of processes under mild reaction conditions. Several lipases, esterases and proteases catalyse with high regioselectivity the esterification or transesterification of sugars in non-aqueous reaction media. Lipases from various origins were largely used for ester synthesis, including phospholipids, either with free or immobilized enzymes [4, 5]. Lipases accept a wide variety of substrates while maintaining their regioselectivity and stereoselectivity [6–8]. Thus, the condensation of secondary amine with oleic acid has been achieved by enzymatic amidification [9]. Recently, esterification of β-CD was performed by Pedersen et al. using thermolysin to obtain a mixture of 1–7 substituted β-CD without any regioselectivity[10].

In this work, considering the insertion of lipidyl-CDs in lipid bilayers, a new class of monosubstituted amphiphilic CDs was synthesized from a single fatty acid or ester via chemo-enzymatic synthesis. The influence of the type of enzyme and of the degree of cyclodextrin methylation is also reported. Finaly, a surprising reaction catalysed by dedicated lipases between acetaldehyde and modified cyclodextrin is described.

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Experimental

Materials

Native β-cyclodextrin was obtained by Wacker Chemicals (Germany). Vinyl laurate (99% purity) was from Fluka Chemie GmbH (Germany) and ethyl caprylate (99.5% purity) from Sigma (USA). Other chemicals were purchased from Sigma and Acros (Belgium). Enzymes were from Biocatalysts Ltd (UK) except Lipozyme from Sigma. All the solvents employed for the reactions were distilled once before use. Deuterated solvents were purchased from Eurisotop (France).

Enzyme preparation and synthesis

6^{I}-amino-6^{I}-deoxy-cyclomaltoheptaose **3** was purified by ion exchange chromatography (BioRad AG MP-50 Resin) and 6^{I}-amino-6^{I}-deoxy-2^{I},3^{I}-di-*O*-methyl-hexakis ($2^{II–VII}$, $3^{II–VII}$, $6^{II–VII}$-Tri-*O*-methyl)cyclomaltoheptaose **4** by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1). Modified cyclodextrin was dissolved with acyl donor as solvent (excess) in presence of suspended enzyme (10–15 mg). Reactions were carried out in an Eppendorf Thermomixer (Brinkmann Instruments, Ontario) with stirring at 1,000 rpm and 40°C under atmospheric pressure during 24 h (see Table 1). The control experiment has been done without enzyme under the same conditions.

For scale up, an orbital shaker was used (109 rpm, 30°C) and 25 mg of enzyme were added to the reaction medium. After 24 h, the enzyme reaction mixture was centrifuged for 5 min at 5,000 rpm to remove biocatalysts. For 100 mg scale reactions, purification was performed to isolate product : partial distillation on reduced pressure to remove fatty acids followed by a column chromatography on silica gel (CH_2Cl_2/MeOH 95:5 then 90:10) monitored by TLC and an ion-exchange chromatography on Dowex 1X8 Cl^- eluted with water.

Analysis

Standard ^{1}H NMR experiments were performed at 500 or 300 MHz using Bruker DRX 500 or AMX 300 spectrometers. Me_4Si was used as an internal standard. Measurements were performed at 25°C. ^{1}H NMR data spectra were collected using 16 K data points. Samples were dissolved in $CDCl_3$. Stepwise control of the reaction has been readily achieved using mass spectrometry ESI-MS analysis in the positive ion mode on ZQ 4000 instrument (Waters-Micromass, UK). Reaction media were diluted in MeOH (0.001 mg/ml) and the solutions directly introduced. In the same conditions, the structure elucidation of the final products was further confirmed by High Resolution Mass

Table 1 Conditions of lipase-catalysed synthesis

Amphiphilic cyclodextrin (1 mg)	Acyl donor (1.2 ml; excess)	Enzyme source	Products	
4	Ethyl caprylate	*Candida rugosa*	**5**	
4	Ethyl caprylate	Lipomod	**5**	
4	Ethyl caprylate	Lipozyme	**5**	
4	Ethyl caprylate	*Mucor javanicus*	**5**	
4	Ethyl caprylate	*Alcagenes* species	**5**	
4	Ethyl caprylate	*Pseudomonas fluorescens*	**5**	
4	Ethyl caprylate	*Rhizopus arrhizus*	**5**	
4	Ethyl caprylate	/	/	
			Open reactor	Closed reactor
4	Vinyl laurate	*Candida rugosa*	**6**	**6**, **7** and **8**
4	Vinyl laurate	Lipomod	**6**	**6**, **7** and **8**
4	Vinyl laurate	Lipozyme	**6**	**6**
4	Vinyl laurate	*Alcaligenes* sp.	**6**	**6**, **8** and **7** (majority)
4	Vinyl laurate	*Pseudomonas fluorescens*	**6**	**6**, **8** and **7** (majority)
4	Vinyl laurate	*Rhizopus niveus*	**6**	**6**
4	Vinyl laurate	*Rhizopus arrhizus*	**6**	**6**, **8** and **7** (majority)
4	Vinyl laurate	*Chromobacterium vicosum*	**6**	**6**
4	Vinyl laurate	*Pancreatic porcine*	**6**	**6**
4	Vinyl laurate	/	**6**	**6**

Spectrometry (HR-MS) using electrospray infusion mode performed in positive mode on a QTOF (Micromass UK) mass spectrometer.

Purity of compounds were controlled by HPLC with a Waters Prep LC 4,000 System chromatograph fitted with DEDL DLS 1,000 (Polymer Laboratories) detector and Prevail C_{18}-bonded silica column (4.6 mm × 250 mm), by elution appropriate solvent at 1 ml/min (Compound (**5**), t_R = 44.3 min : gradient t_0 = 100% H_2O; t_{40} = 100% CH_3CN; Compound (**6**), t_R = 41.6 min : gradient t_0 = 100% H_2O; t_{30} = 100% MeOH). Analytical TLC was performed using Silica Gel 60 F_{254} plates (Merck, Germany) (eluant : CH_2Cl_2/MeOH 9:1) followed by charring with vanillin-H_2SO_4.

Results and discussion

Lipase reactions were performed on **3** and **4,** obtained from β-CD (Scheme 1) using usual method [11–15].

Methylated cyclodextrins were chosen because they are known as good candidates for vector applications. Moreover, they are very soluble in water as well as in organic solvents. Indeed, the methylated cyclodextrins (**4**) can be solubilized in two different acyl donors allowing the use of a solvent-free medium to assess lipase activity for amidification. Oppositely, reactions with **3** were realized in DMSO, Methanol or Acetonitrile because of low solubility of **3** in acyl donors. Nine lipases of diverse origins free or commercially immobilized and chosen for their ability to catalyse transesterification in solvent free medium [16], were tested. The results are summarized in Table 1. Throughout the precise yield or activity were not determine in each cases, all the tested lipases display activity to some degree with **4**. It should be pointed out that, all the attemps performed with **3** failed presumably du to the poor solubility of **3** in medium. A rapid screening using TLC and mass spectroscopy was performed to select good conditions. This method could avoid purification of each test. Systematic studies of enzymatic conditions will be developped subsequently.

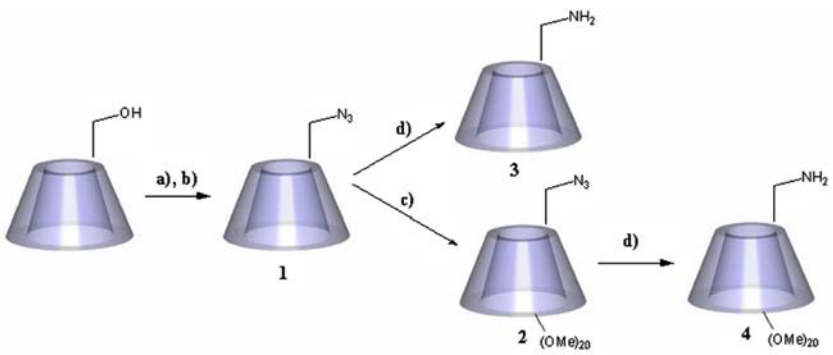
Experiments using partially purified **4** were unsuccessful. Indeed, contamination of triphenylphosphine oxyde inhibited enzymes. It was thus necessary to carefully purified compound **4** with column chromatography on silica gel to carry out lipase reactions. From reaction with ethyl caprylate as substrate and solvent, a compound was detected at *m/z* $[M+Na]^+$ 1562. This compound corresponded to **5** (Scheme 2) indicating that all the tested lipases catalysed the reaction of amine of methylated β-CD **4** with ethyl caprylate.

Without any optimisation of the reaction conditions, the compound **5** was purified and isolated with 28% yield and fully characterized using dedicated bidimensional NMR experiments (Fig. 1) and MS (Fig. 2a).

Using vinyl laurate, several products were detected by MS, particularly: *m/z* $[M+Na]^+$ 1618 (**6**) and $[M+H]^+$1518 (**7**) and 1544 (**8**) (Fig. 3).

The expected product was **6**, i.e. the amidification of the modified methylated β-CD **4** by the laurate moiety. The concomitant synthesis of **7** and **8** let think that vinyl moiety influence the formation of these compounds. As the enzymes catalysed the hydrolysis of vinyl laurate in vinyl alcohol and lauric acid, the vinyl alcohol formed in this reaction led to acetaldehyde which was volatile, an experiment was performed in an open reactor as already described elsewhere [4]. In this case, only **6** was detected. After its purification with 27% yield, the structure was confirmed by NMR and MS (Fig. 2b). This result suggests that acetaldehyde was evaporated during open reactor tests.

Acetaldehyde seemed to have a role in the formation of **7** and **8** and unlike in some other reports [4]



(a)TsCl (8 eq.), $CuSO_4$ (3 eq.), H_20, 4 h at rt then 3 day at 4°C, 30%; (b) LiN_3 (10 eq.), H_20, 5 h at 110°C then 18 h at rt, 100%; (c) CH_3I (185 eq.), NaH (100 eq.), DMF anhydrous, 24 h at rt, 90%; (d) PPh_3 (4 eq.), NH_4OH (20%), DMF, 18 h at rt, 86%;

Scheme 1 Preparation of **3** and **4** cyclodextrins used as acceptor in enzymatic reactions

Scheme 2 New amphiphilic cyclodextrins obtained in one step enzymatic reactions

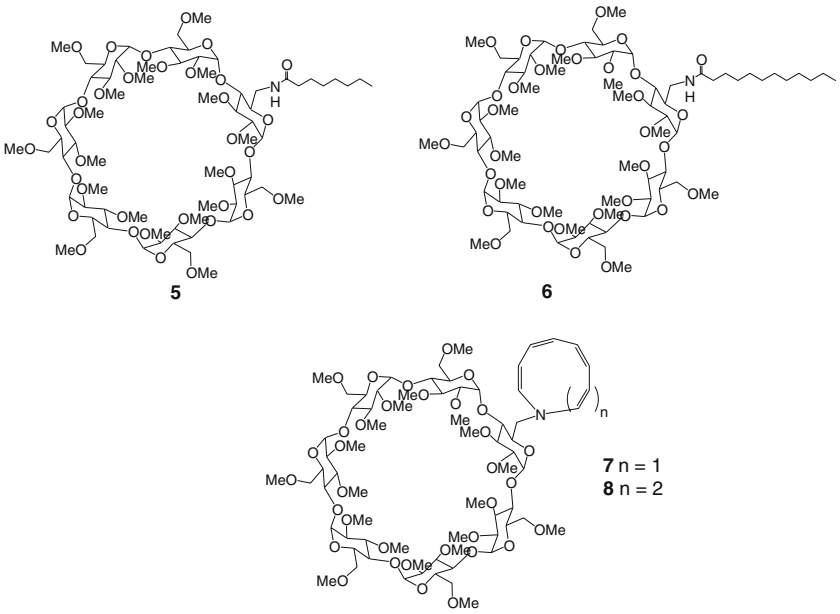


Fig. 1 ^{1}H NMR Spectrum of **5** (500 MHz, $CDCl_3$, 298 K, 5 mM)

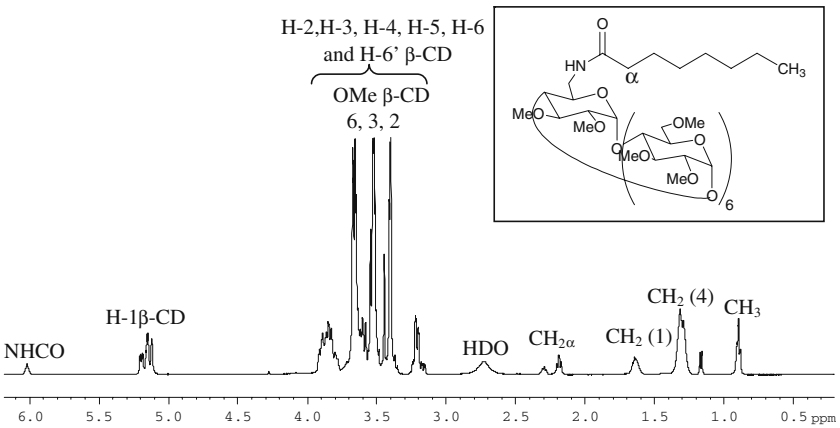
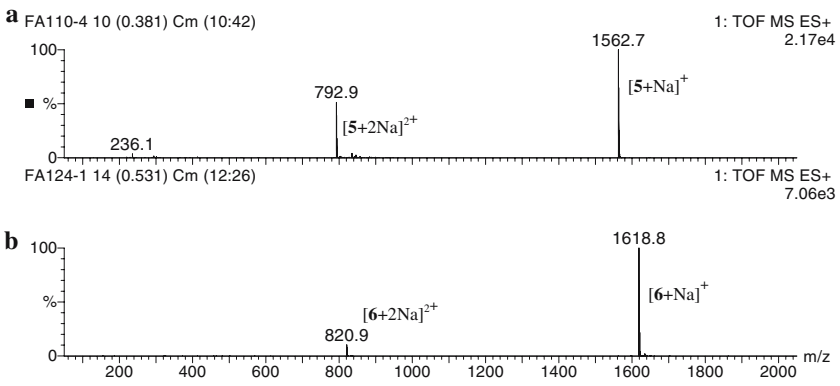


Fig. 2 (**a**) Mass spectrum of **5** (**b**) Mass spectrum of **6** (QTOF experiments)



acetaldehyde does not inactive any of the tested enzymes. These two compounds were difficult to isolate. Structural investigations were carried out on reaction mixtures. Mass spectrometry studies have shown that these compounds had a nitrogen group and seemed to have an added moietie which corresponded to 104 g mol^{-1} and 130 (**7** and **8** respectively). In larger scale, if acetaldehyde and **4** were directly used as

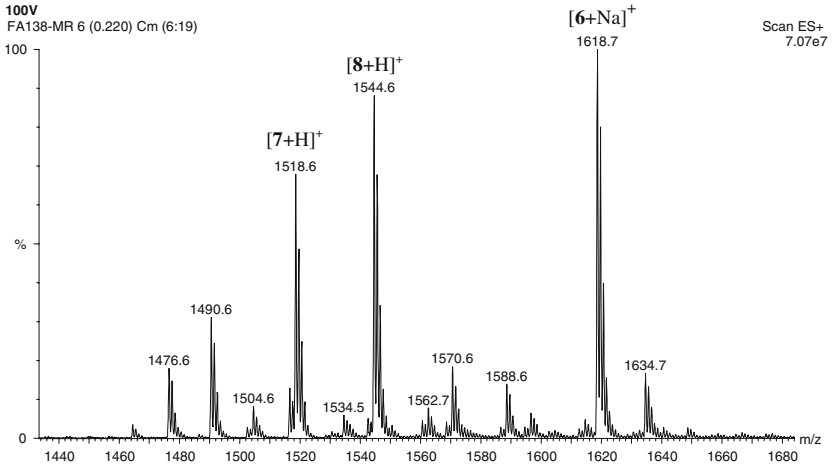


Fig. 3 Mass spectrum (ZQ 4000) of reaction mixture obtained from vinyl laurate, **4** and lipase from Rhizopus arrhizus in closed reactor conditions

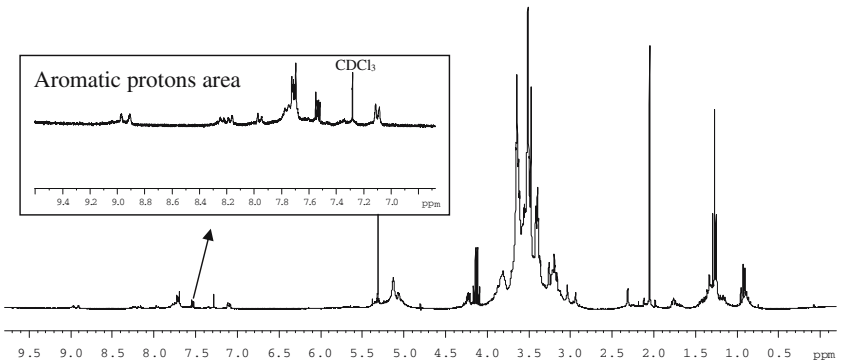


Fig. 4 ^{1}H NMR Spectrum (300 MHz, $CDCl_3$, 298 K, 5 mM) of reaction mixture obtained from vinyl laurate, **4** and lipase from Rhizopus arrhizus in closed reactor conditions

substrates of lipases, the main product formed was **8**. This crude reaction mixture was analyzed by ^{1}H (Fig. 4) and ^{13}C NMR. The presence of aromatic protons and carbons was noteworthy in these two spectra. Informations from NMR and Mass spectroscopy have agreed with aromatic structures: derivatives of methylated-β-CD *N*-nine-membered ring **7** and eleven membered ring **8**. In reaction of **4** with vinyl laurate in absence of enzyme, it should be noted that **7** and **8** were not observed whatever the experimental conditions (open or closed reactors). Finally, attemps to directly use an aliphatic amine as substrate in presence of acetaldehyde and enzyme failed.

These preliminary results have shown that, in presence of lipases, the amine derivative of cyclodextrin **4** could react with acetaldehyde in a controlled ''polymerization'' reaction followed by dehydratation to lead to aromatic compounds in one step. The study of this very interesting reaction is currently in progress in the laboratory.

Conclusion

A very promising method has been developed for the lipase-catalysed synthesis of stable amide bond grafted on methylated cyclodextrin. Indeed, lipase-catalysed of this reaction required only one step, and in a solvent free medium, making easier the purification. The monosubstitution by one fatty acid on methylated cyclodextrin may find applications in cosmetics and pharmaceuticals due to inclusion capacity of CDs and amphiphilic properties. Compounds were fully purified and characterized. Using vinyl laurate as acyl donor, the reaction conditions can be turned to lead to unexpected aromatic compounds. Optimisation and mechanistic studies are carried on extensively in our laboratories.

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