Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 5570

www.rsc.org/obc

PAPER

Polycationic amphiphilic cyclodextrins as gene vectors: effect of the macrocyclic ring size on the DNA complexing and delivery properties†

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Received 24th April 2012, Accepted 31st May 2012
DOI: 10.1039/c2ob25786f

A collection of homologous monodisperse facial amphiphiles consisting of an α-, β- or γ-cyclodextrin (α, β or γCD) platform exposing a multivalent display of cationic groups at the primary rim and bearing hexanoyl chains at the secondary hydroxyls have been prepared to assess the influence of the cyclooligosaccharide core size in their ability to complex, compact and protect pDNA and in the efficiency of the resulting nanocondensates (CDplexes) to deliver DNA into cells and promote transfection in the presence of serum. All the polycationic amphiphilic CDs (paCDs) were able to self-assemble in the presence of the plasmid and produce transfectious nanoparticles at nitrogen/phosphorous ratios ≥5. CDplexes obtained from βCD derivatives generally exhibited higher transfection capabilities, which can be ascribed to their ability to form inclusion complexes with cholesterol, thereby enhancing biological membrane permeability. The presence of thiourea moieties as well as increasing the number of primary amino groups then favour cooperative complexation of the polyphosphate chain, enhancing the stability of the complex and improving transfection. In the α and γCD series, however, only the presence of tertiary amino groups in the cationic clusters translates into a significant improvement of the transfection efficiency, probably by activating endosome escape by the proton sponge mechanism. This set of results illustrates the potential of this strategy for the rational design and optimisation of nonviral gene vectors.

Introduction

Nucleic acids (DNA, siRNA, microRNA, oligonucleotides,…) are a promising source of therapeutics for the treatment of acquired and genetic diseases including various types of cancer, cardiovascular, monogenic and infectious diseases. Due to their poor cellular uptake and rapid degradation in biological media, successful applications critically depend on the development of efficient purpose-conceived carriers that protect and deliver them into their target cells. Because of their natural ability to infect cells, modified viruses have been long considered as the vehicles of choice. However, viral-based vectors display major inherent restrictions, among which a limited DNA carrying capacity, expensive cost and safety concerns such as immunogenic response, toxicity or oncogenicity.[1] During the last three decades, non-viral gene delivery systems have gathered momentum.[2] Most of these non-viral nucleic acid vectors fall within the category of cationic lipids or polymers, featuring functional groups that electrostatically neutralize nucleic acids and cooperatively promote compaction into colloidal nanoparticles termed *lipoplexes* and *polyplexes*, respectively, with increased metabolic stability and membrane permeability. Unfortunately, low efficiency and poor selectivity compared to their viral counterparts limit their application range.[3]

Progress in this field requires a better understanding of the mechanisms involved in cell and systemic traffic of vector : pDNA complexes. Despite their undisputable investigational utility, manipulation of the functional features of many of the first generation non-viral vectors is not an easy task. The intrinsic polydispersity of these materials and their random conformational properties make it difficult to undertake a systematic investigation of the influence of structural modifications on the transfecting properties. Moreover, their generally flexible

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†Electronic supplementary information (ESI) available: NMR spectra of all new compounds. See DOI: 10.1039/c2ob25786f
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character may give rise to self-folding, which decreases the binding ability towards DNA and forces the use of higher vector : nucleic acid ratios to achieve full complexation and protection.[4]

Preorganization of the cationic functional elements onto macrocyclic platforms, such as calixarenes[5] or cyclodextrins[6] (CDs) has the potential to allow control of their spatial orientation and, ultimately, the self-assembling behavior of discrete architectures to produce nanometric objects that can be programmed to complex, compact, deliver and release plasmid DNA in a target cell. Most interestingly, homogeneity can be preserved at the molecular level in structurally related series of compounds by implementing selective chemical functionalization methodologies, offering unprecedented opportunities for structure–activity relationship studies.[7]

In a previous work, we developed a new family of monodisperse polycationic amphiphilic β-cyclodextrin (cyclomaltoheptaose, βCD)-based materials (pa-βCDs), featuring segregated cationic and lipophilic domains, which have been shown to be particularly well-suited for the above channels.[8] A series of pa-βCDs constructs varying in the density and arrangement of the cationic groups and the nature of the linkers were prepared by implementing molecular diversity-oriented approaches and their gene delivery capability evaluated in various cell lines.[9] Facial amphiphilicity and the presence of a belt of hydrogen-bonding centres between the cationic cluster and the CD platform for cooperative and reversible complexation of the polyanionic DNA chain were found to be very favourable features to attain high transgene expression levels and very low toxicity profiles (Fig. 1).

In agreement with the above general observations, the transfection efficiency in murine epithelial COS-7 cells in serum free medium improved by up to 100-fold when going from the cysteaminyl pa-βCD **1β** to the aminoethylthioureido adduct **2β** (Fig. 2). A further 10-fold improvement was achieved for derivatives **3β** and **4β**, displaying a dendritic presentation of the amine functionalities.[9a] Compound **4β** retained high transfection capabilities even in the presence of serum. Most interestingly, the later pa-βCD has proven to be a promising nonviral gene delivery system for *in vivo* applications.[10]

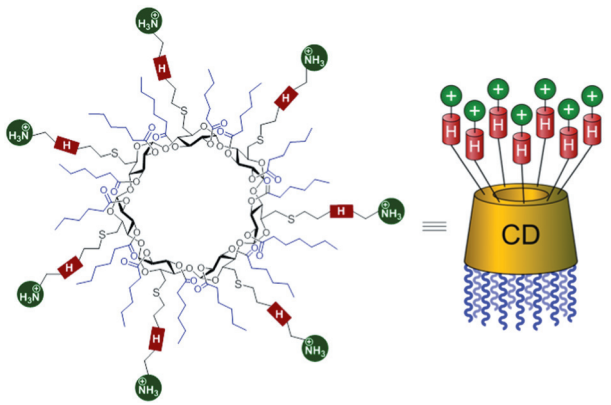


Fig. 1 Schematic representation of the optimal pa-βCD architecture for efficient gene delivery according to previous data. The red rectangles/cylinders represent hydrogen-bonding donating groups (*e.g.* thiourea functionalities), whereas the green circles represent cationic centres, which can be eventually dendronized. The aliphatic chains are depicted in blue.

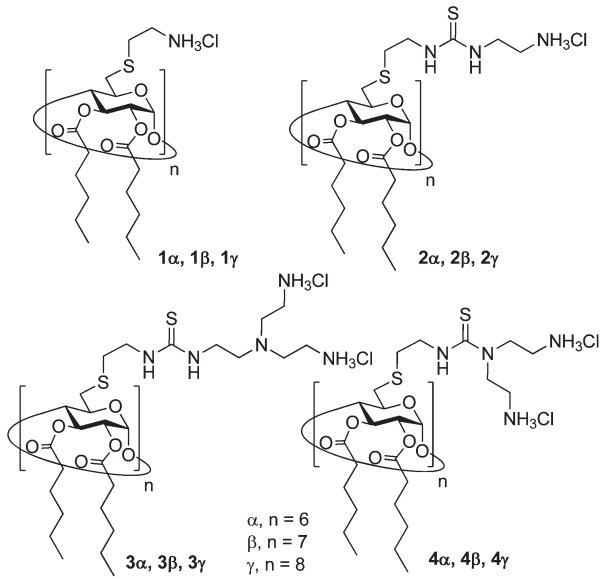


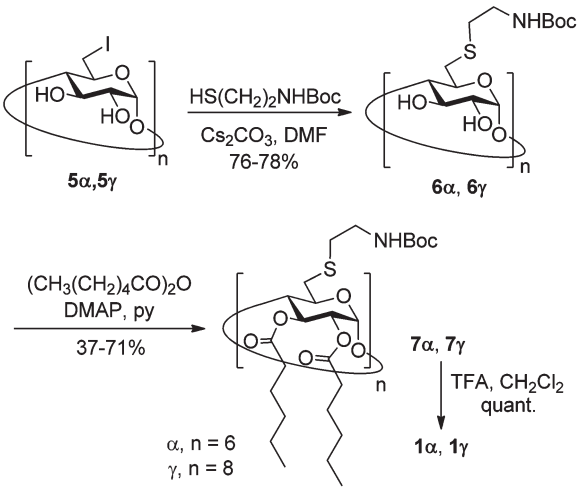
Fig. 2 Structures of the pa-α, -β and -γCD vectors **1α**,**β**,**γ**-**4α**,**β**,**γ**.

Modulating the molecular topology of these preorganized CD-based systems by acting not only on the head and tail groups nature and density, but also on the size of the macrocyclic nucleus, offers further opportunities to optimize both transfection efficiency and cell viability parameters. With this idea in mind, we have now undertaken the synthesis of polycationic amphiphilic derivatives homologous of **1β**–**4β** in the α-cyclodextrin (cyclomaltohexaose, αCD) and γ-cyclodextrin (cyclomaltooctaose, γCD) series, namely the pa-αCDs **1α**–**4α** and the pa-γCDs **1γ**–**4γ** (Fig. 2). Their ability to complex and compact pDNA, the size and surface potential of the resulting nanocomplexes (CDplexes) and their gene delivery and transfection capability in COS-7 cells in the presence of serum, in comparison with data for the βCD-based vectors **1β**–**4β**, are discussed.

Results and discussion

Synthesis

A main problem when facing the synthesis of multifunctional molecular materials is the difficulty in warranting monodispersity at every step, which increases exponentially for high valency platforms such as cyclodextrins. The use of quantitative ligation methods becomes essential to prevent the presence of side-products, often susceptible to positional isomerism, in the reaction mixtures, which generally leads to unaffordable separation problems. For the preparation of the new cysteaminyl paCDs **1α** and **1γ** we have implemented a very efficient three-step synthetic route that involves: (i) nucleophilic displacement of iodide in the corresponding per(6-iodo-6-deoxy)-α and -γCD **5α** and **5γ** by *N*-Boc-protected cysteamine (→**6α** and **6γ**), (ii) hexanoylation of the secondary hydroxyl groups (→**7α** and **7γ**) using hexanoic anhydride/dimethylaminopyridine (DMAP) in *N*,*N*-dimethylformamide (DMF) and (iii) trifluoroacetic acid (TFA)-catalyzed hydrolysis of the carbamate groups (Scheme 1). The hexanoyl



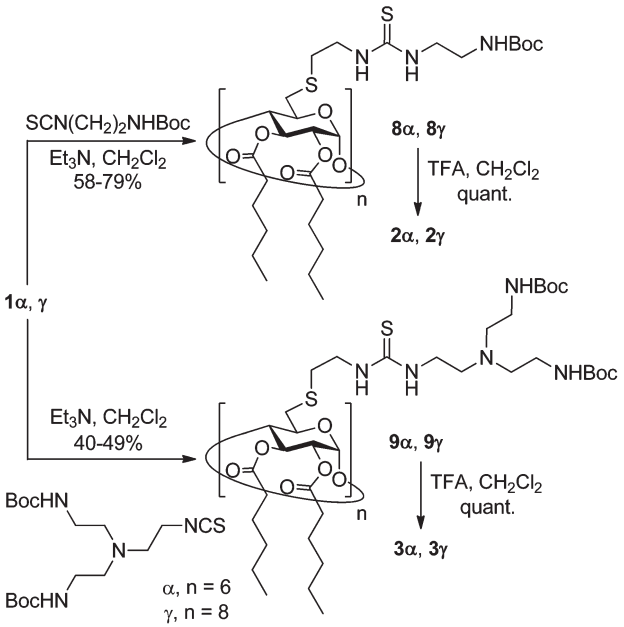
Scheme 1 Synthesis of the cysteaminyl pa-α and -γCD **1α** and **1γ**.

group was chosen in our molecular design since it provided the optimal hydrophilic/hydrophobic balance in our previous studies in the βCD series.[9a] Acylation conditions are particularly critical. The use of other solvents and catalysts (*e.g.* pyridine, triethylamine) or acylation reagents (*e.g.* hexanoyl chloride) led to inhomogeneities due to the presence of under- or oversubstituted compounds.

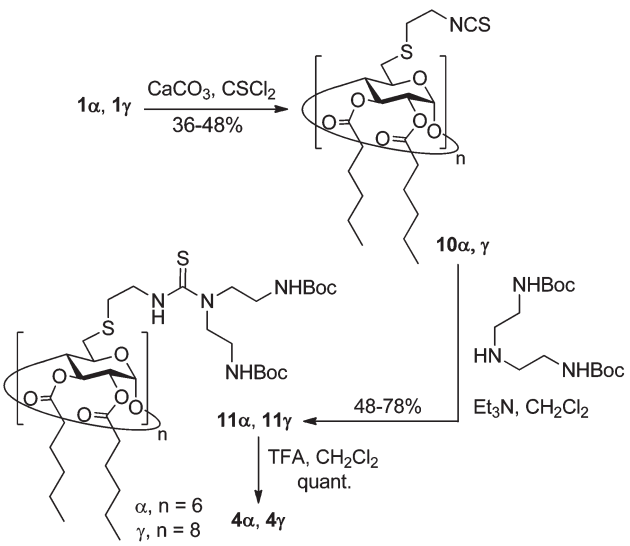
Compounds **1α** and **1γ** are pivotal intermediates in the semi-convergent synthesis of the thiourea adducts **2α**–**4α** and **2γ**–**4γ**. The reaction of amines with isothiocyanates has already proven to be extremely efficient for multiple coupling,[11] including the preparation of hyperbranched CD-conjugates.[12] Thus, compounds **2α** and **2γ**, bearing a single aminoethylthiourea segment per arm, were obtained by thiourea-forming reaction involving the corresponding hexa- and octa-cysteaminyl precursors **1α** and **1γ** and 2-(*N*-*tert*-butoxycarbonylamino)ethyl isothiocyanate (→**8α** and **8γ**; 58–79% yield), followed by acid hydrolysis of the carbamate protecting groups in the thiourea adducts (quantitative). A similar reaction sequence implying multinucleophilic addition of **1α** and **1γ** to 2-[*N*,*N*-bis[2-(*N*-*tert*-butoxycarbonylamino)ethyl]amino]ethyl isothiocyanate (→**9α** and **9γ**; 40–49% yield) and subsequent removal of the Boc protecting groups afforded the dendritic amphiphilic polycationic clusters **3α** and **3γ**, respectively (Scheme 2).

The synthesis of the paCDs **4α** and **4γ**, featuring *N*,*N*′,*N*′-trisubstituted thiourea segments, required the transformation of **1α** and **1γ** into the corresponding polyisothiocyanates **10α** and **10γ**, which was accomplished in 36–48% yield by using thiophosgene as isothiocyanation reagent. Further coupling of **10α** and **10γ** with *N*,*N*-bis[2-(*N*-*tert*-butoxyaminocarbonyl)ethyl]amine (→**11α** and **11γ**, 85%) and final TFA-catalyzed cleavage of the carbamate protecting groups afforded the target derivatives in virtually quantitative yield (Scheme 3).

In all the above syntheses, the final polycationic amphiphilic compounds were obtained in pure form after the last hydrolytic step, with no need for further purification. Prior to physicochemical characterization and biological evaluation, the trifluoroacetate counterion was exchanged by chloride through freeze-drying from diluted HCl to improve sample stability and handling. The ^{1}H and ^{13}C NMR spectra of the carbamate-protected



Scheme 2 Synthesis of the aminoethylthioureido pa-α and -γCDs **2α**, **3α** and **2γ**, **3γ**.



Scheme 3 Synthesis of the aminoethylthioureido pa-αCD and -γCD **4α** and **4γ**.

intermediates and the thiourea adducts showed the typical line broadening associated with slow rotation at the pseudoamide C–N bonds,[11] but they were consistent with the expected C_6- or C_8-symmetric arrangement for α and γCD derivatives, indicative of homogeneous substitution of the CD core. The purity of all compounds was further confirmed by mass spectrometry and combustion analysis.

pDNA complexation and nanoparticle characterization

The capability of the α, β and γCD-based facial amphiphiles **1α**–**4α**, **1β**–**4β**, **1γ**–**4γ** to form nanocondensates with pDNA (a luciferase encoding plasmid of 5739 base pairs used also for

the transfection assays described below) was examined at protonable nitrogen/phosphate group ratios (N/P) 1, 2, 5 and 10. These formulations were characterized by (i) agarose gel electrophoresis for their ability to compact and protect DNA, (ii) dynamic light scattering (DLS) for average hydrodynamic size, (iii) mixed-mode measurement-phase analysis light scattering (M3-PALS) for ζ-potential and (iv) transmission electron microscopy (TEM) for morphology. The pDNA concentration was 200 μM for agarose gel electrophoresis experiments and 60 μM for ζ-potential/size measurements.

Agarose gel electrophoresis retardation experiments (Fig. 3), using ethidium bromide (EB) as staining reagent, demonstrated that the whole set of paCDs studied in this work was able to fully complex pDNA at N/P ≥ 5, as indicated by the absence of free mobile or partially complexed plasmid (no fluorescent staining) in the corresponding lanes. This is the case even at N/P 2 for several of the pa-CDs **1** and **2**. At a given N/P ratio, the CDplexes formulated with these "mono-amino" CDs contain 2- and 3-fold more CD equivalents than those formulated with the "di-amino" CDs **4** and "tri-amino" CDs **3**, respectively, indicating that full protection of pDNA from EB intercalation requires not only a minimum charge ratio but also a minimum molar proportion of condensing/complexing cationic agent. In any case, the cationic density on the facial amphiphilic architecture, *i.e.* the ratio between the number of protonable amino groups *vs*. the number of aliphatic chains connected to the CD platform, seems to be a critical parameter influencing the capacity of paCDs to form well-ordered arrangements in the presence of pDNA and provide efficient protection of the pDNA material from the environment.

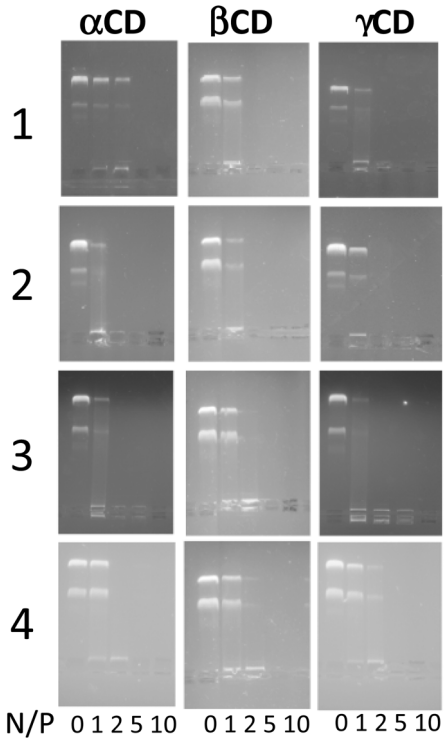


Fig. 3 Electrophoretic mobility of CDplexes formulated from paCDs **1α**,**β**,**γ**-**4α**,**β**,**γ** at different N/P ratios (from left to right: N/P 0, 1, 2, 5 and 10) in agarose gel using ethidium bromide as visualization agent. The DNA concentration was fixed to be 200 μM in phosphate in all cases. The concentration of paCD vector was adjusted to fit the indicated N/P values, considering that the number of protonable (amine) nitrogens is 6, 7, 8 for compounds **1α**,**β**,**γ** and **2α**,**β**,**γ**; 12, 14, 16 for compounds **4α**,**β**,**γ**; and 18, 21, 24 for compounds **3α**,**β**,**γ**, respectively. N/P 1 corresponds then to concentrations of 33.3 (**1α** and **2α**), 28.6 (**1β** and **2β**), 25 (**1γ** and **2γ**), 16.7 (**4α**), 14.4 (**4β**), 12.5 (**4γ**), 11.1 (**3α**), 9.5 (**3β**) and 5.6 μM (**3γ**), for the vectors.

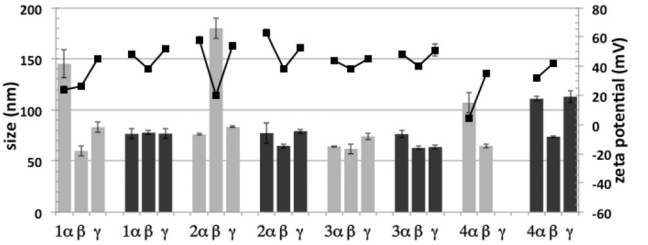


Fig. 4 Size (left axis; bars) and ζ-potential (right axis; black squares and lines) of CDplexes formulated with paCDs **1α**,**β**,**γ**-**4α**,**β**,**γ** determined by DLS and M3-PALS. Grey and black bars correspond to values measured for N/P 5 and 10, respectively. The ζ-potential of **4γ** could not be measured owing to flocculation.

Nanoparticle size was determined by DLS for CDplexes prepared at N/P 5 and 10, for which pDNA is fully complexed (Fig. 4). The formulations prepared from **1α**, **2β**, **4α** and **4γ** at N/P 5 and from **4α** and **4γ** at N/P 10 exhibited relatively large particle sizes (from 110 to 180 nm), similar to those generally obtained for formulations prepared from cationic lipids (lipoplexes) or polymers (polyplexes). In all the other cases, smaller nanoparticles with average hydrodynamic diameters in the 60–80 nm range and quasi-unimodal size distributions were obtained. In contrast to classical lipoplexes, no extrusion process is needed to homogenize particle size distribution. These formulations were further found to display a highly positive ζ-potential (from +20 to +60 mV; Fig. 4), in agreement with full coverage of the DNA chain by paCD units in a well-ordered arrangement.

The low polydispersity and small size of the cationic CDplexes obtained with most of these paCDs confirm the data already evidenced for other members of this gene vector family.[9] Such a behavior has only been observed previously in the case of monomolecular condensation processes occurring upon mixing of DNA with dimerizable polycationic detergents.[13] The TEM images of the CDplexes, formulated at N/P 10, further demonstrated this feature (Fig. 5). In all cases, an ultra-thin structure revealing an alternate arrangement of high (dark) and low (light) electron density regions was observed. The dark regions account for the DNA chain, whereas the light regions probably correspond to bilayers of polycationic amphiphilic CDs.

pDNA delivery and transfection efficiency

The transfection efficiency and cell viability of the CDplexes formulated from pa-α, -β and -γCD vectors at N/P 2, 5 and 10 was evaluated *in vitro* using a luciferase-encoding reporter gene (pDNA = pTG11236, pCMV-SV40-luciferase-SV40pA; 5739 base pairs) on adherent COS-7 cells in the presence of 10% serum and for a low pDNA concentration (0.5 μg of pDNA per

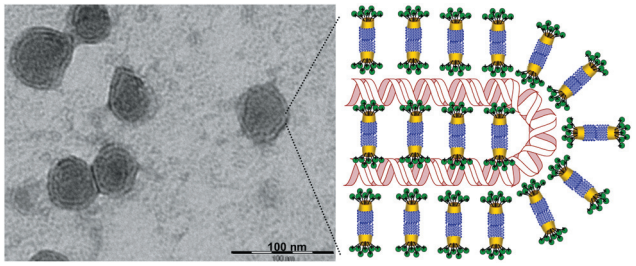


Fig. 5 TEM image of the CDplexes obtained from compound **3α** at N/P 10 showing the snake-like ultra-thin structure and schematic representation of the proposed alternate arrangement of pDNA and paCD bilayers.

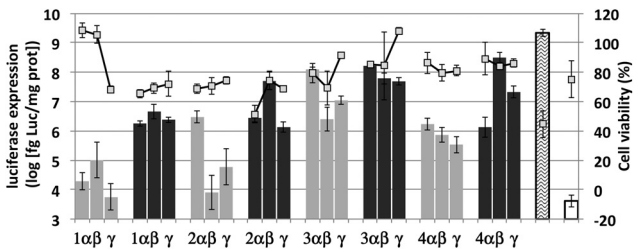


Fig. 6 *In vitro* gene transfection efficiency (left axis; bars) and cell viability (right axis; squares and lines) in COS-7 cells of CDplexes prepared from paCDs **1α**,**β**,**γ**-**4α**,**β**,**γ** at N/P 5 (grey bars) and 10 (black bars) *vs*. data for naked pDNA (white bar) and JetPEI-based polyplexes formulated at N/P 10 (hatched bar).

well; 15 μM phosphate). Polyplexes prepared at N/P 10 from JetPEI (22 kDa), a cationic polymer that ranks among the most efficient nonviral gene delivery systems,[14] and naked pDNA were used as positive and negative controls, respectively. Almost no improvement of transfection with respect to naked pDNA was detected for N/P 2 CDplexes (data not shown). In stark contrast, all paCDs were found to mediate gene transfer and expression under identical conditions at N/P 5 and 10 with much higher efficiencies than naked pDNA (up to 10^5-fold), the performance being generally better at N/P 10 than at N/P 5 (Fig. 6).

Comparison of the transfection efficiency trend in the α, β and γCD series for CDplexes prepared at N/P 10 as a function of the cationic cluster architecture revealed remarkable differences. Thus, the presence of the thiourea moieties in the aminoethylthioureido derivatives **2α** and **2γ** did not result in any significant improvement as compared to the cysteaminyl facial amphiphiles **1α** and **1γ**, while it led to a 15-fold enhancement when going from **1β** to **2β**. Conversely, the presence of the peripheral branched triamino elements in **3α** and **3γ** resulted in a significant increase in the transfection capabilities as compared to **2α** and **2γ**, respectively, but was irrelevant when comparing **3β** and **2β**. Finally, the incorporation of a second aminoethyl segment at the *N*′-thiourea position in **2β** and **2γ** was most beneficial for transfection, as seen when comparing the data for the **2β**/**4β** and **2γ**/**4γ** pairs, while it had no impact in the case of the **2α**/**4α** pair. Overall, compounds **2β**, **3α**–**3γ** and **4β** showed very good transfection abilities, especially considering that the presence of serum is often strongly detrimental for nonviral gene delivery systems. The best performers are the pa-βCD **4β** and the pa-αCD **3α**, with transfection levels only 5- and 10-fold lower compared with JetPEI but with much more favourable cell viability profiles (85% *vs*. 45%). It must be stressed that the transfection data for Jet-PEI polyplexes appear artificially high due to its much higher toxicity. Interestingly, the CDplexes prepared from **3α** were equally efficient when formulated at N/P 5, hence for a two-fold lower amount of CD.

Structure–activity relationships

The ensemble of electrophoretic and nanoparticle characterization data indicate that both the incorporation of the thiourea belt and the multiplication of the cationic centres favor pDNA compaction and protection in a molar basis,[15,16] but no significant differences upon variations in the cyclooligosaccharide size were evidenced. In contrast, the transfection efficiency trends were very different as a function of the α, β or γ-cyclodextrin core size. Considering the data at N/P 10, where full DNA protection is warranted in all cases, the βCD platform generally gave rise to the highest luciferase expression, as seen for the cluster series **1** and, more significantly, **2** and **4** (Fig. 6). This comparative analysis strongly suggests that the presence of the βCD scaffold has an intrinsic favourable impact in the global process leading to protein expression. It is indeed known that βCD derivatives have the capacity to complex cholesterol at the inner hydrophobic cavity, thereby enhancing biological membrane permeability and facilitating cell internalisation and endosome escape capabilities.[17] Actually, βCD has been frequently used as transfection enhancer in gene vector formulations.[7b,18] Although substitution may affect CD inclusion capabilities, previous data support that the cyclooligosaccharide cavity remains accessible in pa-βCDs for size-fitting guests.[9a] The cholesterol-complexing mechanism is absent in the cases of pa-αCD and -γCD derivatives.

The transfection efficiency of pa-βCD-based CDplexes at N/P 10 follows the upward trend **1β** < **2β** ≈ **3β** < **4β**, which probably reflects their relative efficiencies in the reversible complexation of the phosphate groups in the pDNA chain. However, for pa-αCD and -γCD-based CDplexes the transfecting capability follows the trend **1α**,**γ** ≈ **2α**,**γ** ≈ **4α** < **4γ** ≪ **3α**,**γ**. Considering that data for **4γ** are probably overestimated due to the propensity of the resulting CDplexes to flocculate, essentially no improvement in the transfection capacity is obtained after incorporation of the thiourea groups and multiplication of the cationic centres when going from structure **1** to **2** and **4** in these two CD series, in spite of the presumed increase in complexing capabilities. The remarkable enhancement observed for compounds with structure **3**, which are characterized by the presence of a set of tertiary amino groups in addition to the thiourea segments and the peripheral primary amines, probably arise from improvement in the endosome-escaping capabilities through the so-called proton sponge mechanism.[14] Only some of these tertiary basic nitrogen centres are expected to be protonated at physiological pH. Actually, after freeze-drying from dilute hydrochloric acid solutions, only a fraction of the amino groups that corresponds to the fraction of primary amine functionalities in **3α** and **3β** and is 35% higher in the case of **3γ**, appears to stand as the corresponding ammonium chlorides, as seen from microanalytical data (see Experimental). Similarly to the situation encountered in

PEI-based polyplexes, these centres can act as buffering points after acidification at the endosome, resulting in endosome collapse with release of the complexes in the cytoplasm.[14,19]

The slight but statistically significant superiority of the pa-αCD **3α** over the βCD and γCD homologues **3β** and **3γ** might arise from more favourable self-organization capabilities of the smallest αCD-based facial amphiphile onto the pDNA chain. Actually, at N/P 5 the pa-αCD-based CDplexes are significantly more efficient as compared to the nanocomplexes prepared from the pa-βCD and pa-γCD counterparts, excepting for the cysteaminyl clusters **1**, bearing the shorter arms. Elucidating the exact origin of this cyclooligosaccharide size effect in the transfection properties requires further investigation. In any case, taking into consideration that decreasing the amount of vector needed to achieve efficient transfection is generally beneficial for *in vivo* applications, the ensemble of data indicates that compound **3α** is a very promising candidate for gene therapy strategies.

Conclusions

In summary, we have demonstrated that the approach based in the installation of counter-directional multi-head/multi-tail aminothiourea/*O*-hexanoyl domains onto a cyclodextrin platform provides facial amphiphiles with gene delivery capability. Total control of the homogeneity at the molecular level is warranted in homologous series of compounds, allowing reliable structure–activity relationship studies. βCD-based CDplexes formulated at N/P 10 show enhanced transfection efficiencies as compared to CDplexes prepared from αCD or γCD vectors, probably due to the capacity of the βCD cavity to host cholesterol, thereby increasing cell/endosome membrane permeability. Insertion of thiourea segments and increasing the number of primary cationic centers then result in a significant improvement of the transfecting potential by enhancing CDplex stability. The incorporation of tertiary amino groups in the structure is strongly beneficial in the α, β and γCD series, which might be related to the activation of the proton-sponge mechanism for endosome escaping. Regarding the cyclodextrin ring size, the data support that the smallest αCD platform exhibit better complementarity to the pDNA chain, which becomes particularly significant for N/P 5 formulations. The study has allowed identifying promising candidates for further research and provides clues for the rational design of new CD-based gene vectors. The fact that the transfection data have been obtained using 10% serum containing medium points to a certain compatibility with *in vivo* conditions, which has been actually confirmed for the pa-βCD derivative **4β**.[10]

Experimental

General methods

Hexakis(6-deoxy-6-iodo)cyclomaltohexaose (**5α**),[20] octakis(6-deoxy-6-iodo)cyclomaltooctaose (**5γ**),[21] pa-βCDs **1β**–**4β**,[9a] 2-(*N*-*tert*-butoxyaminocarbonyl)ethyl isothiocyanate[22] and 2-[*N*,*N*-bis-[2-(*N*-*tert*-butoxyaminocarbonyl)ethyl]amino]ethyl isothiocyanate[9a] were prepared according to literature procedures. Optical rotations were measured at room temperature in 1 cm or 1 dm tubes on a Jasco P-2000 polarimeter. Ultraviolet-visible (UV) spectra were recorded in 1 cm tubes on a Beckman DU640 UV spectrophotometer. Infrared (IR) spectra were recorded on a Jasco FT/IR 4100-Series spectrophotometer. ^{1}H (and ^{13}C) NMR spectra were recorded at 300 (75.5), 500 (125.7) MHz with Bruker 300 AMX, 500 AMX and 500 DRX instruments. Spectra recorded at 298 K showed broad signals due to slow rotation processes about the NH–C(S) bonds in the NMR time scale. Satisfactory resolutions were achieved after heating above 313 K. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with Kieselgel 60 F254 (E. Merck), with visualization by UV light and by charring with 10% H_2SO_4. Column chromatography was carried out on Silica Gel 60 (E. Merck, 230–400 mesh). ESI mass spectra were recorded in the positive mode on an Esquire 3000 ion-trap mass spectrometer (Bruker Daltonik GmbH). Typically, samples were dissolved in appropriate volumes of deionised water to give sample concentrations of 50 mg L^{-1}. Aliquots were mixed with 25 : 25 : 1 deionised water–methanol–trifluoroacetic acid, generally in a ratio of 1 : 10, to give a total volume of 200 μL. Samples were introduced by direct infusion using a Cole-Parmer syringe at a flow rate of 2 μL min^{-1}. Ions were scanned between 300 and 6000 Da with a scan speed of 13 000 Da s^{-1} at unit resolution using resonance ejection at the multipole resonance of one-third of the radio frequency (Ω = 781.25 kHz). Calibration of the mass spectrometer was performed using ES tuning mix (Hewlett Packard). Recorded data were processed using Bruker Daltonics Esquire 5.0 software (Bruker). Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain).

Preparation of complexes formulated from paCD derivatives and plasmid pTG11236

The plasmid pTG11236 (pCMV-SV40-luciferase-SV40pA) used for the preparation of the DNA complexes and for transfection assay is a plasmid of 5739 bp (base pairs). The amount of compound used was calculated according to the desired DNA concentration of 0.02 mg mL^{-1} or 0.07 mg mL^{-1} for gel electrophoresis experiments (*i.e.* 60 μM or 200 μM phosphate, respectively), the N/P ratio (1, 2, 5 or 10), the molar weight, and the number of protonable nitrogen atoms in the selected CD derivative or cationic polymer (JetPEI). For the preparation of the DNA complexes from CD derivatives and JetPEI, DNA was diluted in HEPES (20 mM, pH 7.4) to a final concentration of 60 μM, then the desired amount of CD derivative was added from 10 or 20 mM stock solution (DMSO) and JetPEI was added from a 0.1 M stock solution (H_2O). For JetPEI polyplexes, DNA was diluted in a 150 mM NaCl solution to a final phosphate concentration of 60 μM, then the desired amount of JetPEI was added from a 7.5 mM NaCl solution. The preparation was vortexed for 2 h and used for characterization or transfection experiments.

Measurement of the size and ζ-potential of the CDplexes

The average size of the CDplexes was measured using a Zetasizer nano (Malvern Instruments, Paris, France) with the following

specification: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; refractive index, 1.33; scattering angle, 173°; λ = 633 nm; temperature, 25 °C. Data were analyzed using the multimodal number distribution software included in the instrument. Results are given as volume distribution of the major population by the mean diameter with its standard deviation. The ζ-potential of the CDplexes was measured using the same apparatus with "mixed-mode measurement" phase analysis light scattering (M3-PALS). M3-PALS consists of both slow field reversal and fast field reversal measurements, hence the name "mixed-mode measurement"; it improves accuracy and resolution. The following specifications were applied: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25 °C. Before each series of experiments, the performance of the instrument was checked with either 90 nm monodisperse latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for ζ-potentials.

Agarose gel electrophoresis

Each CD derivative/DNA sample (20 µL, 0.4 µg of plasmid) was submitted to electrophoresis for about 30 min under 150 V through a 0.8% agarose gel in 1 : 1 : 1 tris(hydroxymethyl)-aminomethane (Tris)–acetate–ethylenediaminetetraacetic acid (EDTA) buffer (TAE buffer) and stained by ethidium bromide (1 µL of a 10 mg mL^{-1} solution for 20 mL of gel). DNA was then visualized after photographing using an UV transilluminator.

In vitro transfection

Twenty-four hours before transfection, COS-7 cells were grown at a density of 2×10^4 cells per well in 96-well plates in Dulbelcco modified Eagle culture medium (DMEM; Gibco-BRL) containing 10% fetal calf serum (FCS; Sigma), glucose (4.5 g L^{-1}), glutamine (2 mM), penicillin (100 units per mL) and 10 mg mL^{-1} gentamycin in a wet (37 °C) and 5% CO_2/95% air atmosphere. The above-described paCD : pDNA (pTG11236) CDplexes and JetPEI : pDNA polyplexes were diluted to 100 µL in DMEM supplemented with 10% FCS so as to have 0.5 µg of pDNA in the well (15 µM phosphate). The culture medium was removed and replaced by these 100 µL of the complexes. After 4 and 24 h, DMEM (50 and 100 µL) supplemented with 30% and 10% FCS, respectively, were added. After 48 h, the transfection was stopped, the culture medium was discarded, and the cells were washed twice with PBS (100 µL) and lysed with lysis buffer (50 µL; Promega, Charbonnières, France). The lysates were frozen at −32 °C before the analysis of luciferase activity. This measurement was performed using a luminometer (GENIOS PRO, Tecan France S.A.) in dynamic mode, for 10 s on the lysis mixture (20 mL) and using the "luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined by the BCA test (Pierce, Montluçon, France). Luciferase activity was calculated as femtograms (fg) of luciferase per mg of protein. The percentage of cell viability was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells ×100. The data were calculated from three or four repetitions in two fully independent experiments (formulation and transfection).

Hexakis[6-(2-*tert*-butoxycarbonylaminoethylthio)]cyclomaltohexaose (6α). To a suspension of **5α** (1.68 g, 1.03 mmol) and Cs_2CO_3 (2.81 g, 8.65 mmol, 1.4 equiv) in dry DMF (10 mL), *tert*-butyl *N*-(2-mercaptoethyl)carbamate (1.46 mL, 8.65 mmol, 1.4 equiv) was added and the reaction mixture was stirred at 60 °C, under Ar atmosphere, for 24 h. The mixture was concentrated up to half of the starting volume, cooled to room temperature, poured into ice-water (50 mL) and stirred for 3 h. The mixture was filtered, washed with H_2O and Et_2O, the solvent removed and the resulting residue was purified by column chromatography (8 : 1 → 4 : 1 CH_2Cl_2 : MeOH). Yield: 1.55 g (78%); R_f 0.28 (8 : 1 CH_2Cl_2 : MeOH); $[\alpha]_D$ +63.4 (*c* 0.98, CH_2Cl_2); IR: ν_{max} 3630, 3334, 2976, 1691, 1515, 1250, 1044 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm) 5.42 (dd, 6 H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 8.5 Hz, H-3), 5.04 (d, 6 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.81 (dd, 6 H, H-2), 4.20 (m, 6 H, H-5), 3.94 (t, 6 H, $J_{4,5}$ = 8.5 Hz, H-4), 3.30 (m, 12 H, CH_2N), 3.07 (s, 12 H, H-6a, H-6b), 2.78–2.68 (m, 12 H, CH_2S), 2.36–2.11 (m, 24 H, $H\text{-}2_{Hex}$), 1.55 (m, 24 H, $H\text{-}3_{Hex}$), 1.44 (s, 54 H, CMe_3), 1.31 (m, 48 H, $H\text{-}4_{Hex}$, $H\text{-}5_{Hex}$), 0.89 (m, 36 H, $H\text{-}6_{Hex}$); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (ppm) 173.4, 171.6 (CO ester), 156.0 (CO carbamate), 96.6 (C-1), 79.3 (C-4), 78.8 (CMe_3), 71.5 (C-3), 71.1 (C-5), 70.6 (C-2), 40.4 (CH_2N), 34.1 ($C\text{-}2_{Hex}$), 34.0 (C-6), 33.8 (CH_2S), 31.4, 31.3 ($C\text{-}4_{Hex}$), 28.5 (CMe_3), 24.4, 24.3 ($C\text{-}3_{Hex}$), 22.4 ($C\text{-}5_{Hex}$), 13.8 ($C\text{-}6_{Hex}$); ESIMS: *m*/*z* 1949.1 $[M + Na]^+$. Anal. Calcd for $C_{73}H_{128}N_6O_{34}S_6$: C, 48.58; H, 7.21; N, 4.36; S, 9.98; found: C, 48.21; H, 7.01; N, 4.09; S, 9.62.

Hexakis[6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose (7α). To a solution of **6α** (1.40 g, 0.77 mmol) in dry pyridine (20 mL) at 0 °C, under Ar atmosphere, DMAP (3.38 g, 27.7 mmol, 3 equiv) and hexanoic anhydride (8.58 mL, 37.1 mmol, 4 equiv) were added and the reaction mixture was stirred at 60 °C for 6 h. The solution was concentrated up to half of the starting volume, MeOH (25 mL) was added dropwise and the mixture was stirred at 40 °C for 18 h. The solution was concentrated and 1 : 1 H_2O : CH_2Cl_2 (100 mL) was added. The organic phase was washed with 2N H_2SO_4 (2 × 50 mL), water (2 × 50 mL) and saturated aqueous $NaHCO_3$ (2 × 50 mL), dried ($MgSO_4$), filtered, exhaustively concentrated under vacuum and purified by column chromatography (1 : 6 → 1 : 4 → 1 : 1 EtOAc : petroleum ether). Yield: 0.89 g (37%); R_f 0.40 (1 : 3 EtOAc : petroleum ether); $[\alpha]_D$ = +73.7 (*c* 1.0, CH_2Cl_2); IR ν_{max} 3627, 2957, 1749, 1508, 1246, 1037 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm) 5.42 (t, 6 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 5.04 (d, 6 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.81 (dd, 6H, H-2), 4.20 (m, 6 H, H-5), 3.94 (t, 6 H, $J_{4,5}$ = 9.3 Hz, H-4), 3.30 (m, 12 H, CH_2N), 3.07 (s, 12 H, H-6a, H-6b), 2.78–2.68 (m, 12 H, CH_2S), 2.36–2.11 (m, 24 H, $H\text{-}2_{Hex}$), 1.55 (m, 24 H, $H\text{-}3_{Hex}$), 1.44 (s, 54 H, CMe_3) 1.31 (m, 48 H, $H\text{-}4_{Hex}$, $H\text{-}5_{Hex}$), 0.89 (m, 36 H, $H\text{-}6_{Hex}$); ^{13}C NMR (125.7 MHz, $CDCl_3$, 298 K) δ (ppm) 173.4, 171.6 (CO ester), 156.0 (CO carbamate), 96.6 (C-1), 79.3 (C-4), 78.8 (CMe_3), 71.5 (C-3), 71.1 (C-5), 70.6 (C-2), 40.4 (CH_2N), 34.1 ($C\text{-}2_{Hex}$),

34.0 (C-6), 33.8 (CH_2S), 31.4, 31.3 (C-4_{Hex}), 28.5 (CMe_3), 24.4, 24.3 (C-3_{Hex}), 22.4 (C-5_{Hex}), 13.8 (C-6_{Hex}); ESIMS: *m/z* 3126.9 $[M + Na]^+$. Anal. Calcd for $C_{149}H_{256}N_6O_{48}S_6$: C, 57.88; H, 8.35; N, 2.72; S, 6.22; found: C, 57.71; H, 8.19; N, 2.48; S, 5.87.

Hexakis[6-(2-aminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose hexahydrochloride (1α). Compound **7α** (327 mg, 0.105 mmol) was treated with 1 : 1 TFA : CH_2Cl_2 (2 mL) at room temperature for 2 h. Then solvent was evaporated and acid traces removed by co-evaporation with water. The residue was dissolved in 10 : 1 H_2O : HCl 0.1 M and freeze-dried to yield quantitatively **1α**. Yield: 286 mg; $[\alpha]_D$ +62.4 (*c* 0.99, MeOH); ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.49 (bs, 6 H, H-3), 5.16 (bs, 6 H, H-1), 4.84 (bs, 6 H, H-2), 4.29 (bs, 6 H, H-5), 3.99 (bs, 6 H, H-4), 3.30 (bs, 6 H, H-6a), 3.26 (t, 12 H, $^3J_{H,H}$ = 6.0 Hz, CH_2N), 3.15 (bs, 6 H, H-6b), 3.05 (m, 12 H, CH_2S), 2.42, 2.31 (bs, 24 H, H-2_{Hex}), 1.64 (t, 24 H, $^3J_{H,H}$ = 7.0 Hz, H-3_{Hex}), 1.38 (m, 48 H, H-4_{Hex}, H-5_{Hex}), 0.96 (m, 36 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ (ppm) 174.7, 173.4 (CO ester), 98.1 (C-1), 80.8 (C-4), 73.4 (C-5), 72.0 (C-3, C-2), 40.4 (CH_2N), 35.2 (C-6), 34.9 (C-2_{Hex}), 32.5 (C-4_{Hex}), 31.9 (CH_2S), 25.6 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.2 (C-6_{Hex}); ESIMS: *m/z* 2505.2 $[M + H]^+$. Anal. Calcd for $C_{120}H_{216}Cl_6N_6O_{36}S_6$: C, 52.91; H, 7.99; N, 3.09; S, 7.06; found: C, 52.57; H, 7.68; N, 2.72; S, 6.75.

Octakis[6-(2-*tert*-butoxycarbonylaminoethylthio)]cyclomaltooctaose (6γ). To a suspension of **5γ** (2 g, 0.92 mmol) and Cs_2CO_3 (3.35 g, 10.3 mmol, 1.4 equiv) in dry DMF (10 mL), 2-(Boc-amino)ethanethiol (1.74 mL, 10.3 mmol, 1.4 equiv) was added and the reaction mixture was stirred at 60 °C under Ar for 24 h. Work-up as described for **6α** and purification by column chromatography (7 : 1 → 5 : 1 → 4 : 1 → 2 : 1 CH_2Cl_2 : MeOH) gave **6γ**. Yield: 1.82 g (76%); R_f 0.33 (50 : 10 : 1 CH_2Cl_2 : MeOH : H_2O); $[\alpha]_D$ +67.5 (*c* 0.97, 16 : 1 MeOH : CH_2Cl_2); IR ν_{max} 3626, 3327, 2977, 1689, 1513, 1159, 1036 cm^{-1}; ^{1}H NMR (500 MHz, 5 : 1 CD_3OD–$CDCl_3$) δ (ppm) 5.17 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.08 (m, 8 H, H-5), 3.96 (t, 8 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.68 (dd, 8 H, H-2), 3.64 (t, 8 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.47 (t, 16 H, $^3J_{H,H}$ = 6.0 Hz, CH_2N), 3.33 (d, 8 H, $J_{6a,6b}$ = 12.5 Hz, H-6a), 3.05 (m, 8 H, H-6b), 2.92 (m, 16 H, CH_2S), 1.60 (s, 72 H, CMe_3); ^{13}C NMR (125.7 MHz, 5 : 1 CD_3OD–$CDCl_3$) δ (ppm) 158.4 (CO carbamate), 104.2 (C-1), 86.1 (C-4), 80.7 (CMe_3), 74.8 (C-2), 74.6 (C-3), 73.8 (C-5), 41.7 (CH_2N), 34.8 (C-6), 34.7 (CH_2S), 29.5 (CMe_3). ESIMS *m/z* 3593.0 $[M + Na]^+$. Anal. Calcd for $C_{104}H_{180}N_8O_{48}S_8$: C, 48.58; H, 7.21; N, 4.36; S, 9.98; found: C, 48.32; H, 7.12; N, 4.10; S, 9.70.

Octakis[6-(2-*tert*-butoxycarbonylaminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose (7γ). To a solution of **6γ** (1.73 g, 0.67 mmol) in dry pyridine (20 mL) under Ar atmosphere, DMAP (3.92 g, 32.1 mmol, 3 equiv) was added. Hexanoic anhydride (10 mL, 42.9 mmol, 4 equiv) was added dropwise and the reaction mixture was stirred at 60 °C under Ar for 17 h. Work-up as described for **7α** and purification by column chromatography (1 : 6 → 1 : 4 → 1 : 3 EtOAc : petroleum ether) afforded **7γ**. Yield: 1.96 g (71%); R_f 0.27 (1 : 3 EtOAc : petroleum ether); $[\alpha]_D$ +90.3 (*c* 0.99, CH_2Cl_2); IR ν_{max} 3627, 2957, 1748, 1508, 1246, 1037 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 5.30 (t, 8 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3), 5.16 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.71 (dd, 8 H, H-2), 4.10 (m, 8 H, H-5), 3.72 (t, 8 H, $J_{4,5}$ = 9.0 Hz, H-4), 3.31 (m, 16 H, CH_2N), 3.11 (bd, 8 H, $J_{6a,6b}$ = 11.0 Hz, H-6a), 3.02 (m, 8 H, H-6b), 2.78–2.71 (m, 16 H, CH_2S), 2.42–2.10 (m, 32 H, H-2_{Hex}), 1.57 (m, 32 H, H-3_{Hex}), 1.44 (s, 72 H, CMe_3) 1.30 (m, 64 H, H-4_{Hex}, H-5_{Hex}), 0.89 (m, 48 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (ppm) 173.4, 171.7 (CO ester), 156.0 (CO carbamate), 96.3 (C-1), 79.2 (C-4), 77.9 (CMe_3), 71.4 (C-5), 70.8 (C-2), 69.8 (C-3), 40.0 (CH_2N), 33.9, 33.8 (C-2_{Hex}), 33.6 (CH_2S), 33.5 (C-6), 31.4, 31.2 (C-4_{Hex}), 28.5 (CMe_3), 24.4, 24.3 (C-3_{Hex}), 22.4 (C-5_{Hex}), 14.2, 13.9 (C-6_{Hex}); ESIMS *m/z* 4162.7 $[M + Na]^+$. Anal. Calcd for $C_{200}H_{344}N_8O_{64}S_8$: C, 58.00; H, 8.37; N, 2.71; S, 6.19; found: C, 57.66; H, 8.22; N, 2.48; S, 5.81.

Octakis[6-(2-aminoethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose octahydrochloride (1γ). Compound **1γ** was obtained by treatment of **7γ** (254 mg, 0.061 mmol) with 1 : 1 TFA : CH_2Cl_2 (2 mL) as described for **1α**. Yield: 213 mg (96%); $[\alpha]_D$ +65.4 (*c* 1.28, MeOH); IR ν_{max} 3624, 2957, 1747, 1674, 1166, 1036 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.39 (t, 8 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.27 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.85 (dd, 8 H, H-2), 4.12 (t, 8 H, $J_{4,5}$ = 9.5 Hz, H-5), 3.86 (t, 8 H, H-4), 3.31–3.20 (m, 24 H, H-6a, CH_2N), 3.13–3.03 (m, 24 H, H-6b, CH_2S), 2.55–2.26 (m, 32 H, H-2_{Hex}), 1.65 (m, 32 H, H-3_{Hex}), 1.39 (m, 64 H, H-4_{Hex}, H-5_{Hex}), 0.97 (m, 48 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ (ppm) 174.9, 174.3 (CO ester), 97.9 (C-1), 79.7 (C-4), 73.9 (C-5), 72.2 (C-2), 71.4 (C-3), 40.4 (CH_2N), 35.2, 35.0 (C-2_{Hex}), 34.3 (C-6), 32.6 (C-4_{Hex}), 31.5 (CH_2S), 25.6 (C-3_{Hex}), 23.6, 23.4 (C-5_{Hex}), 14.3 (C-6_{Hex}); ESIMS: *m/z* 3338.9 $[M + H]^+$. Anal. Calcd for $C_{160}H_{288}Cl_8N_8O_{48}S_8$: C, 52.91; H, 7.99; N, 3.09; S, 7.06; found: C, 50.53; H, 7.60; N, 2.76; S, 6.71.

Hexakis[6-(2-(*N*′-(2-*tert*-butoxycarbonylaminoethyl)thioureido)-ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose (8α). To a solution of **1α** (158 mg, 0.058 mmol) in CH_2Cl_2 (5 mL), Et_3N (0.15 mL) was added and the mixture was stirred for 10 minutes. Then, 2-(*tert*-butoxycarbonylamino)ethyl isothiocyanate (76 mg, 0.038 mmol, 1.08 equiv) was added and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum and the residue purified by column chromatography (1 : 1 → 5 : 4 → 3 : 2 → 2 : 1 EtOAc : petroleum). Yield: 125 mg (58%); R_f 0.39 (9 : 1 CH_2Cl_2 : MeOH); $[\alpha]_D$ +75.5 (*c* 1.06, CH_2Cl_2); UV (CH_2Cl_2) 248 nm (ε_{mM} 89.6); IR ν_{max} 3628, 3312, 2957, 1749, 1684, 1246, 1037 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.47 (dd, 6 H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 5.15 (d, 6 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.83 (dd, 6 H, H-2), 4.30 (m, 6 H, H-5), 4.02 (t, 6 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.77 (bs, 12 H, SCH_2CH_2), 3.60 (bs, 12 H, CH_2CH_2NHBoc), 3.32 (m, 6 H, H-6a), 3.29 (t, 12 H, $^3J_{H,H}$ = 6.0 Hz, CH_2NHBoc), 3.25 (m, 6 H, H-6b), 3.00–2.90 (m, 12 H, CH_2S), 2.47–2.25 (m, 24 H, H-2_{Hex}), 1.64 (m, 24 H, H-3_{Hex}), 1.48 (s, 54 H, CMe_3) 1.39 (m, 48 H, H-4_{Hex}, H-5_{Hex}), 0.96 (m, 36 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ 182.4 (CS), 173.4, 172.0

(CO ester), 157.3 (CO carbamate), 96.5 (C-1), 79.1 (CMe$_3$), 78.8 (C-4), 71.6 (C-5), 71.0 (C-3), 70.7 (C-2), 42.8 (CH$_2$NHCS), 39.7 (CH$_2$NHBoc), 34.1 (C-6), 33.9, 33.6 (C-2$_{Hex}$), 32.7 (CH$_2$S), 31.2, 31.4 (C-4$_{Hex}$), 27.5 (CMe_3), 24.2 (C-3$_{Hex}$), 22.1 (C-5$_{Hex}$), 12.9 (C-6$_{Hex}$); ESIMS m/z 3739.6 $[M + Na]^+$. Anal. Calcd for $C_{168}H_{294}N_{18}O_{48}S_{12}$: C, 54.26; H, 7.97; N, 6.78; found: C, 54.12; H, 8.02; N, 6.54.

Hexakis[6-(2-(*N*′-(2-aminoethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose hexahydrochloride (2α). Compound **2α** was obtained by treatment of **8α** (64 mg, 0.017 mmol) with 1 : 1 TFA : CH_2Cl_2 (2 mL) as described for **1α**. Yield: 60 mg; $[\alpha]_D$ +65.3 (c 1.05, MeOH); UV (MeOH) 244 nm (ε_{mM} 70.7); IR ν_{max} 3236, 2955, 1748, 1552, 1160, 1037 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD, 313 K) δ (ppm) 5.24 (dd, 6 H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 8.4 Hz, H-3), 4.91 (d, 6 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.58 (dd, 6 H, H-2), 4.06 (m, 6H, H-5), 3.78 (t, 6 H, $J_{4,5}$ = 8.4 Hz, H-4), 3.67 (t, 12 H, $^3J_{H,H}$ = 6.0 Hz, CH_2CH$_2$NH$_3$Cl), 3.54 (m, 12 H, SCH$_2$CH_2), 3.04 (m, 12 H, H-6a, H-6b), 2.99 (t, 12 H, CH_2NH$_3$Cl), 2.75–2.67 (m, 12 H, CH$_2$S), 2.25–2.00 (m, 24 H, H-2$_{Hex}$), 1.41 (m, 24 H, H-3$_{Hex}$), 1.13 (m, 48 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.72 (m, 36 H, H-6$_{Hex}$); ^{13}C NMR (75.5 MHz, CD_3OD, 313 K): δ (ppm) 183.5 (CS), 173.5, 172.0 (CO ester), 96.5 (C-1), 78.8 (C-4), 71.7 (C-5), 70.9 (C-3), 70.7 (C-2), 44.0 (CH$_2$CH$_2$NH$_3$Cl), 41.1 (SCH$_2$$CH_2$), 39.6 (CH$_2NH_3$Cl), 34.1 (C-6), 33.8, 33.6 (C-2$_{Hex}$), 32.6 (CH$_2$S), 31.3, 31.1 (C-4$_{Hex}$), 24.3, 24.2 (C-3$_{Hex}$), 22.2, 22.1 (C-5$_{Hex}$), 12.9 (C-6$_{Hex}$); ESIMS m/z 3117.2 $[M + H]^+$. Anal. Calcd for $C_{138}H_{252}Cl_6N_{18}O_{36}S_{12}$: C, 49.67; H, 7.61; N, 7.56; S, 11.53; found: C, 49.36; H, 7.43; N, 7.31; S, 11.20.

Octakis[6-(2-(*N*′-(2-*tert*-butoxycarbonylaminoethyl)thioureido)-ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose (8γ). To a solution of **1γ** (367 mg, 0.101 mmol) in CH_2Cl_2 (10 mL), Et_3N (0.15 mL) was added and the mixture was stirred for 10 minutes. Then, 2-(*tert*-butoxycarbonylamino)ethyl isothiocyanate (132 mg, 0.654 mmol, 1.08 equiv) was added and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum and the residue purified by column chromatography (40 : 1 → 20 : 1 CH_2Cl_2 : MeOH). Yield: 396 mg (79%); R_f 0.26 (20 : 1 CH_2Cl_2 : MeOH); $[\alpha]_D$ +77.0 (c 1.0, CH_2Cl_2); UV (CH_2Cl_2) 249 nm (ε_{mM} 58.0); IR ν_{max} 3627, 2958, 2931, 1748, 1265, 1037 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 333 K) δ (ppm) 5.46 (t, 8 H, $J_{2,3}$ = $J_{3,4}$ = 10.5 Hz, H-3), 5.24 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.80 (dd, 8 H, H-2), 4.24 (bs, 8 H, H-5), 3.85 (t, 8 H, $J_{4,5}$ = 9.0 Hz, H-4), 3.78 (bt, 16 H, SCH$_2$CH_2), 3.61 (m, 16 H, CH_2CH$_2$NHBoc), 3.30 (m, 24 H, CH_2NHBoc, H-6a), 3.19 (bs, 6 H, H-6b), 3.02–2.90 (m, 16 H, CH$_2$S), 2.47–2.26 (m, 32 H, H-2$_{Hex}$), 1.65 (t, 32 H, $^3J_{H,H}$ = 6.5 Hz, H-3$_{Hex}$), 1.48 (s, 72 H, CMe$_3$) 1.37 (m, 64 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.96 (m, 48 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, CD_3OD, 333 K) δ (ppm) 182.6 (CS), 173.3, 172.6 (CO ester), 157.1 (CO carbamate), 96.3 (C-1), 79.0 (C-4, CMe$_3$), 71.2 (C-2), 70.6 (C-5), 70.3 (C-3), 43.9 (2 × CH_2NHCS), 39.8 (CH$_2$NHBoc), 34.0 (C-6), 33.6 (C-2$_{Hex}$), 32.4 (CH$_2$S), 31.3, 31.1 (C-4$_{Hex}$), 27.6 (CMe_3), 24.2 (C-3$_{Hex}$), 22.0 (C-5$_{Hex}$), 12.9 (C-6$_{Hex}$); ESIMS: m/z 2521.6 $[M + 2K]^{2+}$. Anal. Calcd for $C_{224}H_{392}N_{24}O_{64}S_{16}$: C, 54.26; H, 7.97; N, 6.78; S, 10.35; found: C, 53.91; H, 7.67; N, 6.42; S, 9.98.

Octakis[6-(2-(*N*′-(2-aminoethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose octahydrochloride (2γ). Compound **2γ** was obtained by treatment of **8γ** (206 mg, 0.042 mmol) with 1 : 1 TFA : CH_2Cl_2 (1 : 1, 2 mL) as described for **1α**. Yield: 186 mg; $[\alpha]_D$ +65.0 (c 0.91, MeOH); UV (MeOH): 245 nm (ε_{mM} 87.2); IR ν_{max} 3627, 2929, 1743, 1551, 1037 cm^{-1}; ^{1}H NMR (500 MHz, Me_2SO-d_6, 333 K) δ (ppm) 7.97 (bs, 3 H, NH$_3$Cl), 7.78 (m, 1 H, NH), 7.71 (m, 1H, NH), 5.28 (t, 12 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, 8 H, H-3), 5.13 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.73 (dd, 8 H, H-2), 4.12 (m, 8 H, H-5), 3.85 (t, 8 H, $J_{4,5}$ = 9.0 Hz, H-4), 3.69 (t, 16 H, $^3J_{H,H}$ = 5.5 Hz, CH_2CH$_2$NH$_3$Cl), 3.63 (m, 16 H, SCH$_2$CH_2), 3.11 (m, 16 H, H-6a, H-6b), 3.01 (t, 16 H, CH_2NH$_3$Cl), 2.83–2.80 (m, 16 H, CH$_2$S), 2.42–2.18 (m, 32 H, H-2$_{Hex}$), 1.55 (m, 32 H, H-3$_{Hex}$), 1.30 (m, 64 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.88 (m, 48 H, H-6$_{Hex}$); ^{13}C NMR (100.6 MHz, Me_2SO-d_6, 333 K) δ (ppm) 183.5 (CS), 172.8, 172.1 (CO ester), 96.6 (C-1), 78.2 (C-4), 71.9 (C-5), 70.9 (C-2), 70.4 (C-3), 44.2 (SCH$_2$$CH_2$), 41.7 ($CH_2CH_2NH_3$Cl), 38.9 (CH$_2NH_3$Cl), 33.8 (C-6), 32.9 (CH$_2$S), 33.8 (C-2$_{Hex}$, C-6), 31.2 (C-4$_{Hex}$), 24.3 (C-3$_{Hex}$), 22.2 (C-5$_{Hex}$), 13.9 (C-6$_{Hex}$); ESIMS m/z 2078.5 $[M + 2 H]^{2+}$. Anal. Calcd for $C_{184}H_{336}Cl_8N_{24}O_{48}S_{16}$: C, 49.67; H, 7.61; N, 7.56; S, 11.53; found: C, 49.29; H, 7.33; N, 7.19; S, 11.13.

Hexakis[6-(2-(*N*′-(2-(*N*,*N*-di-(2-(*N*-*tert*-butoxycarbonylamino)-ethyl)amino)ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose (9α). To a solution of **1α** (84 mg, 0.031 mmol) in CH_2Cl_2 (4 mL), Et_3N (0.05 mL, 2 equiv) was added and the mixture was stirred for 10 minutes. Then, 2-[*N*,*N*-bis[2-(*N*-*tert*-butoxyaminocarbonyl)ethyl]amino]ethyl isothiocyanate (79 mg, 0.204 mmol, 1.1 equiv) was added and the reaction mixture was stirred at room temperature for 5 days. The solvent was removed under vacuum and the residue purified by column chromatography (30 : 1 → 20 : 1 → 9 : 1 CH_2Cl_2 : MeOH). Yield: 60 mg (40%); R_f 0.51 (9 : 1 CH_2Cl_2–MeOH); $[\alpha]_D$ +39.9 (c 1.0, CH_2Cl_2); UV (CH_2Cl_2) 246 nm (ε_{mM} 68.8); IR ν_{max} 2957, 2926, 1751, 1686, 1248, 1165, 1039 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 323 K) δ (ppm) 5.44 (t, 6 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3), 5.19 (bs, 12 H, NHBoc), 5.05 (d, 12 H, $J_{1,2}$ = 3.5 Hz, 6 H, H-1), 4.85 (dd, 6 H, H-2), 4.23 (m, 6 H, H-5), 4.00 (t, 6 H, $J_{4,5}$ = 9.0 Hz, H-4), 3.78 (m, 12 H, SCH$_2$CH_2), 3.58 (m, 12 H, CH$_2$CH_2NHCS), 3.17 (m, 36 H, H-6a, H-6b, CH_2NHBoc), 2.98–2.81 (m, 12 H, CH$_2$S), 2.70 (m, 12 H, CH_2CH$_2$NHCS), 2.61 (m, 24 H, CH_2CH$_2$NHBoc), 2.39–2.15 (m, 24 H, H-2$_{Hex}$), 1.59 (m, 24 H, H-3$_{Hex}$), 1.47 (s, 54 H, CMe$_3$), 1.33 (m, 48 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.93 (m, 36 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, $CDCl_3$, 323 K) δ (ppm) 182.6 (CS), 173.3, 171.6 (CO ester), 156.4 (CO carbamate), 96.8 (C-1), 79.4 (C-4), 79.0 (CMe$_3$), 71.7 (C-3), 71.4 (C-5), 70.7 (C-2), 54.7 (CH$_2$CH$_2$-NHBoc), 54.0 (NCH$_2$CH$_2$NHCS), 44.1 (SCH$_2$$CH_2$), 42.5 (NCH$_2$-$CH_2$NHCS), 39.1 ($CH_2$NHBoc), 34.1 (C-6), 33.8 (C-2$_{Hex}$), 33.2 (CH$_2$S), 31.4, 31.3 (C-4$_{Hex}$), 28.4 (CMe_3), 24.3 (C-3$_{Hex}$), 22.3 (C-5$_{Hex}$), 13.7 (C-6$_{Hex}$); ESIMS m/z 2460.9 $[M + Na + Cu]^{2+}$. Anal. Calcd for $C_{222}H_{402}N_{30}O_{60}S_{12}$ (4836.51): C, 55.13; H, 8.38; N, 8.69; S, 7.96; found: C, 55.30; H, 8.24; N, 8.32; S, 7.58.

Hexakis[6-(2-(*N*′-(2-(*N*,*N*-bis-(2-aminoethyl)amino)ethyl)thio-ureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose dodecahydro-chloride (3α). Compound **3α** was obtained by treatment of **9α**

(33 mg, 0.005 mmol) with 1 : 1 TFA : CH_2Cl_2 (2 mL) as described for **1α**. Yield: 28 mg; $[\alpha]_D$ +65.4 (*c* 0.95, MeOH); ^{1}H NMR (500 MHz, CD_3OD, 313 K): δ (ppm) 5.47 (t, 6 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 5.15 (d, 6 H, $J_{1,2}$ = 3.3 Hz, H-1), 4.82 (dd, 6 H, H-2), 4.30 (m, 6 H, H-5), 4.02 (t, 6 H, H-4), 3.79 (bs, 12 H, SCH_2CH_2), 3.71 (bs, 12 H, NCH_2CH_2NHCS), 3.29 (m, 12 H, H-6a, H-6b), 3.16 (m, 24 H, CH_2NH_3Cl), 2.97 (m, 12 H, CH_2S), 2.90 (m, 12 H, $CH_2CH_2NH_3Cl$), 2.90 (m, 24 H, CH_2CH_2NHCS), 2.45–2.23 (m, 24 H, H-2_{Hex}), 1.63 (m, 24 H, H-3_{Hex}), 1.38 (m, 48 H, H-4_{Hex}, H-5_{Hex}), 0.95 (m, 36 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 313 K) δ (ppm) 182.7 (CS), 173.5, 172.0 (CO ester), 96.5 (C-1), 78.8 (C-4), 71.6 (C-5), 70.9 (C-3), 70.7 (C-2), 52.4 (CH_2CH_2NHCS), 51.3 ($CH_2CH_2NH_3Cl$), 43.9 (SCH_2CH_2), 41.2 (NCH_2CH_2NHCS), 37.3 (CH_2NH_3Cl), 34.1 (C-6), 33.8, 33.1 (C-2_{Hex}), 32.7 (CH_2S), 31.2, 31.1 (C-4_{Hex}), 24.2 (C-3_{Hex}), 22.1 (C-5_{Hex}), 12.9 (C-6_{Hex}). Anal. Calcd for $C_{162}H_{318}Cl_{12}N_{30}O_{36}S_{12}$: C, 47.78; H, 7.87; N, 10.32; S, 9.45; found: C, 47.40; H, 7.58; N, 10.03; S, 9.11.

Octakis[6-(2-(*N*′-(2-(*N*,*N*-di-(2-(*N*-*tert*-butoxycarbonylamino)-ethyl)amino)ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose (9γ). To a solution of **1γ** (107 mg, 0.029 mmol) in CH_2Cl_2 (4 mL), Et_3N (0.064 mL, 2 equiv) was added and stirred for 10 minutes. Then, 2-[*N*,*N*-bis[2-(*N*-*tert*-butoxyaminocarbonyl)-ethyl]amino]ethyl isothiocyanate (99 mg, 0.255 mmol, 1.08 equiv) was added and the reaction mixture was stirred at room temperature for 6 days. The solvent was removed under vacuum and the residue purified by column chromatography (18 : 1 CH_2Cl_2 : MeOH). Yield: 100 mg (49%); R_f 0.61 (9 : 1 CH_2Cl_2 : MeOH); $[\alpha]_D$ +56.7 (*c* 1.0, CH_2Cl_2); UV (CH_2Cl_2) 248 nm (ε_{mM} 96.2); IR ν_{max} 3628, 2957, 1749, 1685, 1247, 1038 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 323 K) δ (ppm) 5.32 (t, 8 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 5.24, (bs, 16 H, NHBoc), 5.16 (d, 8 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.76 (dd, 8H, H-2), 4.12 (m, 8 H, H-5), 3.79 (m, 24 H, H-4, SCH_2CH_2), 3.57 (m, 16 H, CH_2CH_2NHCS), 3.18 (m, 40 H, H-6a, CH_2NHBoc), 3.11 (m, 8 H, H-6b), 2.95–2.82 (m, 16 H, CH_2S), 2.71 (m, 16 H, NCH_2CH_2NHCS), 2.62 (m, 32 H, CH_2CH_2NHBoc), 2.50–2.13 (m, 32 H, H-2_{Hex}), 1.62 (m, 32 H, H-3_{Hex}), 1.47 (s, 72 H, CMe_3), 1.35 (m, 64 H, H-4_{Hex}, H-5_{Hex}), 0.93 (m, 48 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, $CDCl_3$, 323 K) δ (ppm) 182.7 (CS), 173.3, 171.6 (CO ester), 156.4 (CO carbamate), 96.5 (C-1), 79.4 (C-4), 78.2 (CMe_3), 71.7 (C-5), 70.8 (C-2), 70.1 (C-3), 54.7 (CH_2CH_2NHBoc), 54.0 (CH_2CH_2NHCS), 44.0 (SCH_2CH_2), 42.5 (CH_2CH_2NHCS), 39.1 (CH_2NHBoc), 34.0 (C-2_{Hex}), 33.8 (C-6), 33.1 (CH_2S), 31.4, 31.3 (C-4_{Hex}), 28.5 (CMe_3), 24.3 (C-3_{Hex}), 22.6 (C-5_{Hex}), 13.8 (C-6_{Hex}); ESIMS *m*/*z* 3246.5 [M + 2Na]$^{2+}$. Anal. Calcd for $C_{296}H_{536}N_{40}O_{80}S_{16}$: C, 55.13; H, 8.38; N, 8.69; S, 7.96; found: C, 55.02; H, 8.10; N, 8.29; S, 7.63.

Octakis[6-(2-(*N*′-(2-(*N*,*N*-bis-(2-aminoethyl)amino)ethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose hexadecahydrochloride (3γ). Compound **3γ** was obtained by treatment of **9γ** (23 mg, 0.004 mmol) with 1 : 1 TFA : CH_2Cl_2 (2 mL) as described for **1α**. Yield: 19 mg; $[\alpha]_D$ = +55.0 (*c* 0.95, MeOH); IR ν_{max} 3244, 3039, 2956, 2926, 2859, 1747, 1680, 1167, 1038 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 333 K) δ (ppm) 5.38 (m, 8 H, H-3), 5.24 (bs, 8 H, H-1), 4.82 (m, 8H, H-2), 4.21 (m, 8 H, H-5), 3.00 (m, 8 H, H-4), 3.81 (m, 16 H, SCH_2CH_2), 3.75 (m, 16 H, CH_2CH_2NHCS), 3.28 (m, 16 H, H-6a, H-6b), 3.20 (m, 32 H, CH_2NH_3Cl), 3.00 (m, 16 H, CH_2S), 2.97 (m, 32 H, $CH_2CH_2NH_3Cl$), 2.85 (m, 16 H, CH_2CH_2NHCS), 2.53–2.21 (m, 32 H, H-2_{Hex}), 1.66 (m, 32 H, H-3_{Hex}), 1.39 (m, 64 H, H-4_{Hex}, H-5_{Hex}), 0.96 (m, 48 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 333 K): δ (ppm) 182.6 (CS), 173.3, 172.3 (CO ester), 96.6 (C-1), 79.2 (C-4), 71.9 (C-5), 70.9 (C-2), 70.3 (C-3), 52.6 (CH_2CH_2NHCS), 51.4 ($CH_2CH_2NH_3Cl$), 44.0 (SCH_2CH_2), 41.5 (NCH_2CH_2NHCS), 37.5 (CH_2NH_3Cl), 33.9 (C-6), 33.7 (C-2_{Hex}), 32.8 (CH_2S), 31.2, 31.1 (C-4_{Hex}), 24.2 (C-3_{Hex}), 22.0 (C-5_{Hex}), 12.9 (C-6_{Hex}). Anal. Calcd for $C_{216}H_{424}Cl_{16}N_{40}O_{48}S_{16}$·3 HCl: C, 46.83; H, 7.77; N, 10.11; S, 9.26; found: C, 46.82; H, 7.82; N, 10.05; S, 9.09.

Hexakis[2,3-di-*O*-hexanoyl-6-(2-isothiocyanatoethylthio)]cyclomaltohexaose (10α). To a solution of **1α** (137 mg, 0.050 mmol) in CH_2Cl_2 (5 mL), triethylamine (42 μL, 1 equiv), $CaCO_3$ (121 mg, 4 equiv) and thiophosgene (46 μL, 2 equiv) were added and the reaction mixture was stirred at room temperature for 1 hour. The phases were separated and the aqueous phase was extracted with CH_2Cl_2. The organic phase was washed with water, dried ($MgSO_4$), filtered and the solvent removed. The residue was purified by column chromatography (1 : 4 EtOAc : petroleum ether). Yield: 67 mg (48%); R_f 0.40 (1 : 3 EtOAc : petroleum ether); $[\alpha]_D$ +83.8 (*c* 1.0, CH_2Cl_2); IR ν_{max} 2956, 2109, 1747, 1266, 1037 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 323 K) δ (ppm) 5.42 (t, 6 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.16 (bs, 6 H, H-1), 4.79 (dd, 6 H, $J_{1,2}$ = 3.5 Hz , H-2), 4.33 (bs, 6 H, H-5), 3.90 (t, 6 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.82 (m, 12 H, CH_2NCS), 3.14 (m, 6H, H-6a), 3.08–2.96 (m, 18 H, CH_2S, H-6b), 2.38–2.19 (m, 24 H, H-2_{Hex}), 1.60 (m, 24 H, H-3_{Hex}), 1.34 (m, 48 H, H-4_{Hex}, H-5_{Hex}), 0.91 (m, 36 H, H-6_{Hex}); ^{13}C NMR (75.5 MHz, $CDCl_3$, 323 K) δ (ppm) 173.5, 171.8 (CO ester), 131.9 (NCS), 95.7 (C-1), 77.2 (C-4), 71.2 (C-3, C-5), 70.6 (C-2), 45.2 (CH_2NCS), 34.4 (C-6), 34.1, 33.8 (C-2_{Hex}), 33.2 (CH_2S), 31.4, 31.3 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.4 (C-5_{Hex}), 13.9 (C-6_{Hex}); ESIMS *m*/*z* 2779.6 [M + Na]$^+$. Anal. Calcd for $C_{126}H_{198}N_6O_{36}S_{12}$: C, 54.88; H, 7.24; N, 3.05; S, 13.95; found: C, 55.07; H, 7.39; N, 2.87; S, 13.50.

Hexakis[6-(2-(*N*′,*N*′-bis-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)-thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose (11α). To a solution of **10α** (62 mg, 0.022 mmol) in CH_2Cl_2 (3 mL), Et_3N (0.02 mL, 1.1 equiv) and bis[2-(*tert*-butoxycarbonylamino) ethyl]amine (45 mg, 0.148 mmol, 1.1 equiv) were added and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum and the residue purified by column chromatography (1 : 1 → 2 : 1 EtOAc : petroleum ether). Yield: 81 mg (78%); R_f 0.20 (1 : 1 EtOAc : petroleum ether); $[\alpha]_D$ +43.7 (*c* 1, CH_2Cl_2); IR ν_{max} 3300, 2957, 2929, 1749, 1246, 1037 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.49 (t, 6 H, $J_{2,3}$ = $J_{3,4}$ = 8.0 Hz, H-3), 5.16 (d, 6 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.82 (dd, 6 H, H-2), 4.32 (m, 6 H, H-5), 4.05 (t, 6 H, $J_{4,5}$ = 8.0 Hz, H-4), 3.93 (m, 12 H, SCH_2CH_2), 3.80 (bs, 24 H, CH_2CH_2NHBoc), 3.48 (m, 6 H, H-6a), 3.34 (m, 24 H, CH_2NHBoc), 3.29 (m, 6 H, H-6b), 3.05 (m, 12 H, CH_2S), 2.47–2.25 (m, 24 H, H-2_{Hex}), 1.66 (m, 24 H, H-3_{Hex}), 1.49 (s, 108 H, CMe_3), 1.38 (m, 48 H, H-4_{Hex}, H-5_{Hex}), 0.96 (m, 36 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ (ppm) 184.0

(CS), 175.7, 174.3 (CO ester), 159.5 (CO carbamate), 98.7 (C-1), 80.9 (*C*Me$_3$), 81.5 (C-4), 73.7 (C-5), 73.5 (C-3), 73.0 (C-2), 52.9 (*C*H$_2$CH$_2$NHBoc), 48.0 (*CH$_2$*CH$_2$S), 40.4 (*C*H$_2$NHBoc), 36.5 (C-6), 36.1, 35.9 (C-2$_{Hex}$), 34.9 (CH$_2$S), 33.5, 33.4 (C-4$_{Hex}$), 29.9 (C*Me$_3$*), 26.5 (C-3$_{Hex}$), 24.3 (C-5$_{Hex}$), 15.1 (C-6$_{Hex}$); ESIMS *m/z* 4600.2 [M + Na]$^+$. Anal. Calcd for $C_{210}H_{372}N_{24}O_{60}S_{12}$ (4578.11): C, 55.09; H, 8.19; N, 7.34; S, 8.40; found: C, 54.98; H, 8.18; N, 7.41; S, 8.38.

Hexakis[6-(2-(*N*′,*N*′-bis-(2-aminoethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltohexaose dodecahydrochloride (4α). Compound **4α** was obtained by treatment of **11α** (11 mg, 0.024 mmol) with 1 : 1 TFA : CH_2Cl_2 (2.2 mL) as described for **1α**. Yield: 92 mg; $[\alpha]_D$ +38.5 (*c* 1, CH_2Cl_2); IR ν_{max} 3384, 2956, 2925, 1748, 1159, 1038 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.48 (t, 6 H, $J_{2,3} = J_{3,4} = 8.0$ Hz, H-3), 5.16 (d, 6 H, $J_{1,2} = 3.5$ Hz, H-1), 4. 38 (dd, 6 H, H-2), 4.31 (m, 6 H, H-5), 4.16 (m, 24 H, C*H*$_2$CH$_2$NH$_3$Cl), 4.05 (t, 6 H, $J_{4,5}$ = 8.0 Hz, H-4), 3.94 (m, 24 H, SCH$_2$C*H*$_2$), 3.36 (m, 6 H, H-6a), 3.34 (m, 24 H, C*H*$_2$NH$_3$Cl), 3.26 (m, 6 H, H-6b), 3.07 (m, 12 H, CH$_2$S), 2.47–2.25 (m, 24 H, H-2$_{Hex}$), 1.65 (m, 24 H, H-3$_{Hex}$), 1.38 (m, 48 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.96 (m, 36 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ (ppm) 185.0 (CS), 175.8, 174.3 (CO ester), 98.8 (C-1), 81.0 (C-4), 73.9 (C-5), 73.3 (C-3), 73.0 (C-2), 48.3 (*C*H$_2$CH$_2$NH$_3$Cl), 47.4 (SCH$_2$*C*H$_2$), 39.3 (CH$_2$NH$_3$Cl), 36.6 (C-6), 36.1, 35.9 (C-2$_{Hex}$), 34.5 (CH$_2$S), 33.5, 33.4 (C-4$_{Hex}$), 26.4 (C-3$_{Hex}$), 24.3 (C-5$_{Hex}$), 15.1 (C-6$_{Hex}$); ESIMS *m/z* 4600.2 [M + Na]$^+$. Anal. Calcd for $C_{150}H_{288}Cl_{12}N_{24}O_{36}S_{12}$: C, 47.23; H, 7.61; N, 8.81; S, 10.09; found: C, 47.41; H, 7.84; N, 8.60; S, 9.63.

Octakis[2,3-di-*O*-hexanoyl-6-(2-isothiocyanatoethylthio)]cyclomaltooctaose (10γ). To a solution of, **1γ** (250 mg, 0.069 mmol) in 1 : 1 CH_2Cl_2 : H_2O (50 mL), Et$_3$N (77 μL, 1 equiv), $CaCO_3$ (220 mg, 4 equiv) and $SCCl_2$ (84 μL, 2 equiv) were added and the reaction mixture was stirred at room temperature for 1 h. The two phases were separated and the aqueous phase was extracted with CH_2Cl_2. The organic phase was washed with water, dried ($MgSO_4$), filtered and the solvent removed under vacuum. The residue was purified by column chromatography (1 : 4 EtOAc : petroleum ether). Yield: 90 mg (36%); R_f 0.42 (1 : 4 EtOAc : petroleum ether); $[\alpha]_D$ +76.0 (*c* 1.1, CH_2Cl_2); IR ν_{max} 2956, 2080, 1742, 1036 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, 313 K) δ (ppm) 5.32 (t, 8 H, $J_{2,3} = J_{3,4} = 11.0$ Hz, H-3), 5.15 (d, 8 H, $J_{1,2} = 4.5$ Hz, H-1), 4.77 (dd, 8H, H-2), 4.12 (m, 8 H, H-5), 3.80 (m, 16 H, CH$_2$NCS), 3.78 (m, 8 H, H-4), 3.19 (bd, 8H, H-6a), 3.10–3.05 (m, 8 H, H-6b), 3.03–2.91 (m, 16 H, CH$_2$S), 2.43–2.13 (m, 32 H, H-2$_{Hex}$), 1.59 (m, 32 H, H-3$_{Hex}$), 1.31 (m, 64 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.90 (m, 48 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K) δ (ppm) 173.2, 171.8 (CO ester), 132.8 (NCS), 96.6 (C-1), 78.0 (C-4), 71.7 (C-5), 70.6 (C-2), 69.9 (C-3), 45.6 (*C*H$_2$NCS), 34.1 (C-6), 34.0 (C-2$_{Hex}$), 33.8 (CH$_2$S), 31.4, 31.3 (C-4$_{Hex}$), 24.4, 24.3 (C-3$_{Hex}$), 22.3 (C-5$_{Hex}$), 13.8 (C-6$_{Hex}$); ESIMS *m/z* 3702.0 [M + Na]$^+$, 1861.7 [M + 2 Na]$^{2+}$. Anal. Calcd for $C_{168}H_{264}N_8O_{48}S_{16}$: C, 54.88; H, 7.24; N, 3.05; S, 13.95; found: C, 55.10; H, 7.39; N, 2.93; S, 13.91.

Octakis[6-(2-(*N*′,*N*′-bis-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)-thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose (11γ). To a solution of **10γ** (215 mg, 0.058 mmol) in CH_2Cl_2 (8 mL), Et$_3$N (0.07 mL, 1.1 equiv) and bis[2-(*tert*-butoxycarbonylamino) ethyl]amine (157 mg, 0.516 mmol, 1.1 equiv) were added and the reaction mixture was stirred at room temperature overnight. The solvent was removed under vacuum and the residue was purified by column chromatography (40 : 1 → 30 : 1 → 20 : 1 CH_2Cl_2 : MeOH). Yield: 170 mg (48%); R_f 0.31 (20 : 1 CH_2Cl_2 : MeOH); $[\alpha]_D$ +53.3 (*c* 1.0, CH_2Cl_2); IR ν_{max} 3328, 2957, 2927, 1750, 1687, 1165 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 323 K) δ (ppm) 5.38 (t, 8 H, $J_{2,3} = J_{3,4} = 8.3$ Hz, H-3), 5.124 (bd, 8 H, $J_{1,2} = 3.4$ Hz, H-1), 4.80 (dd, 8 H, H-2), 4.20 (m, 8 H, H-5), 3.91 (m, 24 H, H-4, SCH$_2$C*H*$_2$), 3.79 (bs, 32 H, C*H*$_2$CH$_2$NHBoc), 3.33 (bs, 32 H, C*H*$_2$NHBoc), 3.32 (m, 8 H, H-6a), 3.25 (m, 8 H, H-6b), 3.02 (bs, 16 H, CH$_2$S), 2.51–2.22 (m, 32 H, H-2$_{Hex}$), 1.66 (m, 32 H, H-3$_{Hex}$), 1.48 (s, 144 H, CMe$_3$) 1.39 (m, 64 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.95 (m, 48 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, CD_3OD, 323 K) δ (ppm) 182.4 (CS), 173.4, 172.0 (CO ester), 157.3 (CO carbamate), 96.5 (C-1), 79.1 (*C*Me$_3$), 78.8 (C-4), 71.6 (C-5), 71.0 (C-3), 70.7 (C-2), 42.8 (2 × *C*H$_2$NHCS), 39.7 (*C*H$_2$NHBoc), 34.1 (C-6), 33.9, 33.6 (C-2$_{Hex}$), 32.7 (CH$_2$S), 31.2, 31.4 (C-4$_{Hex}$), 27.5 (C*Me$_3$*), 24.2 (C-3$_{Hex}$), 22.1 (C-5$_{Hex}$), 12.9 (C-6$_{Hex}$); ESIMS *m/z* 3115.8 [M + 2 Cu]$^{2+}$. Anal. Calcd for $C_{250}H_{496}N_{32}O_{80}S_{16}$ (6104.14): C, 55.09; H, 8.19; N, 7.34; S, 8.40; found: C, 55.15; H, 8.41; N, 7.65; S, 8.64.

Octakis[6-(2-(*N*′,*N*′-bis-(2-aminoethyl)thioureido)ethylthio)-2,3-di-*O*-hexanoyl]cyclomaltooctaose octahydrochloride (4γ). Compound **4γ** was obtained by treatment of **11γ** (130 mg, 0.021 mmol) with 1 : 1 TFA : CH_2Cl_2 (4 mL) as described for **1α**. Yield: 108 mg; $[\alpha]_D$ +65.4 (*c* 0.95, MeOH); ^{1}H NMR (500 MHz, Me_2SO-d_6, 333 K) δ (ppm) 8.27 (bs, 56 H, NH$_3$Cl, NHCS), 5.26 (bs, 8 H, H-3), 5.15 (bs, 8 H, H-1), 4.72 (m, 8H, H-2), 4.11 (m, 8 H, H-5), 4.00 (bs, 32 H, C*H*$_2$CH$_2$NH$_3$Cl), 3.75 (bs, 8 H, H-4), 3.69 (bs, 16 H, SCH$_2$C*H*$_2$), 3.14 (bs, 48 H, C*H*$_2$ NH$_3$Cl, H-6ab), 2.90 (bs, 16 H, CH$_2$S), 2.19 (m, 16 H, H-2a$_{Hex}$), 2.09 (m, 16 H, H-2b$_{Hex}$), 1.55 (m, 32 H, H-3$_{Hex}$), 1.28 (m, 64 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.88 (m, 48 H, H-6$_{Hex}$); ^{13}C NMR (125.7 MHz, Me_2SO-d_6, 333 K) δ (ppm) 184.9 (CS), 175.5, 174.6 (CO ester), 99.3 (C-1), 80.8 (C-4), 74.5, 73.5, 73.1 (C-2, C-3, C-5), 50.9 (*C*H$_2$CH$_2$NH$_3$Cl), 48.7 (*C*H$_2$CH$_2$S) 39.7 (2 *C*H$_2$NH$_3$Cl), 38.6 (C-6), 36.5 (C-2$_{Hex}$), 35.1 (CH$_2$S), 33.8 (C-4$_{Hex}$), 26.9 (C-3$_{Hex}$), 24.9 (C-3$_{Hex}$), 16.6 (C-6$_{Hex}$); ESIMS *m/z* 3338.9 [M + H]$^+$. Anal. Calcd for $C_{200}H_{384}Cl_{16}N_{32}O_{48}S_{16}$ (5085.66): C, 47.23; H, 7.61; N, 8.81; S, 10.09; found: C, 46.91; H, 7.40; N, 8.43; S, 9.68.

Acknowledgements

The Spanish Ministerio de Economía y Competitividad (contract numbers CTQ2010-15848 and SAF2010-15670; cofinanced with the Fondo Europeo de Desarrollo Regional FEDER), the Junta de Andalucía, the CSIC, the CNRS, and FUSINT (CNR project) are thanked for funding. The CITIUS (Universidad de Sevilla) is also acknowledged for technical assistance.

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