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Polycationic Amphiphilic Cyclodextrins for Gene Delivery: Synthesis and Effect of Structural Modifications on Plasmid DNA Complex Stability, Cytotoxicity, and Gene Expression

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Abstract: A molecular-diversity-oriented approach for the preparation of well-defined polycationic amphiphilic cyclodextrins (paCDs) as gene-delivery systems is reported. The synthetic strategy takes advantage of the differential reactivity of primary versus secondary hydroxyl groups on the CD torus to regioselectively decorate each rim with cationic elements and lipophilic tails, respectively. Both the charge density and the hydrophobic–hydrophilic balance can be finely tuned in a highly symmetrical architecture that is reminiscent of both cationic lipids and cationic polymers, the two most prominent types of nonviral gene vectors. The monodisperse nature of paCDs and the modularity of the synthetic scheme are particularly well suited for structure–activity relationship studies. Gel electrophoresis revealed that paCDs self-assemble in the presence of plasmid DNA (pDNA) to provide homogeneous, stable nanoparticles (CDplexes) of 70–150 nm that fully protect pDNA from the environment. The transfection efficiency of the resulting CDplexes has been investigated in vitro on BNL-CL2 and COS-7 cell lines in the absence and presence of serum and found to be intimately dependent on architectural features. Facial amphiphilicity and the presence of a cluster of cationic and hydrogen-bonding centers for cooperative and reversible complexation of the polyanionic DNA chain is crucial to attain high transgene expression levels with very low toxicity profiles. Further enhancement of gene expression, eventually overcoming that of polyplexes from commercial polyethyleneimine (PEI) polymers (22 kDa), is achieved by building up space-oriented dendritic polycationic constructs.

Keywords: amphiphiles · cyclodextrins · gene delivery · nanoparticles · self-assembly

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Introduction

The successful delivery of therapeutic genes to target cells and their availability at the intracellular site of action are crucial requirements for gene therapy. Naked plasmid DNA (pDNA) has been shown to transfect cells both in vitro and in vivo; however, the transfection efficiency is generally low due to rapid degradation by serum nucleases and limited membrane-permeation potential.[1] In an effort to increase transfection capabilities, much research has been carried out on the development of effective carrier vectors, traditionally divided into viral and nonviral systems, that compact, protect, and deliver genes.[2] The U.S. Food and Drug Administration (FDA) has not approved any viral-vector-based therapeutics up to date due to immunogenicity, oncogenicity, and potential virus recombination concerns.[3] Nonviral DNA condensing agents are currently the subject of increasing attention because of their relative safety and simplicity of use, though they are still far from achieving the gene expression efficiencies of viral systems.[4] The design of artificial carriers that could prove as efficient as their viral counterparts but that are safer to use, homogeneous, nonimmunogenic, and more readily adapted to tailor-made elaboration represents the ultimate challenge for the future development of gene therapy.

Most of the currently available nonviral gene vectors belong to two main groups: cationic lipids and cationic polymers.[5] Both types of compounds can condense pDNA into multimolecular complexes, named lipoplexes or polyplexes, respectively, that show a range of sizes and physicochemical properties in solution. Lipoplexes and polyplexes usually are positively charged particles that efficiently enter the cell after binding to negatively charged proteoglycans on the outer face of the membrane, thereby resulting in improved pharmacokinetics and pharmacodynamics and, eventually, active intracellular delivery.[6] Further functional elements can be incorporated onto the vector or the preformed pDNA–vector complex by means of covalent or supramolecular interactions to help the system to overcome the cellular barriers and the immune defense mechanisms, thus preventing undesired side effects or targeting specific tissues.[7] The cyclodextrin (CD)-containing polycationic polymers developed by Pun and Davis are paradigmatic examples in this respect.[8] The cyclomaltooligosaccharide framework imparts biocompatibility, significantly alleviating the cytotoxicity of cationic polymers[9] both in vitro and in vivo,[10] behaves as a transfection enhancer, and offers unique possibilities associated with the intrinsic molecular inclusion capabilities of the CD cavity,[11,12] which has been translated into therapeutic applications.[13] Polyrotaxanes consisting of cationic CD derivatives threaded onto poly(ethyleneglycol) (PEG) or poly(ethyleneimine) (PEI) chains have also shown efficient gene-delivery capabilities.[14] Unfortunately, the essentially polydisperse nature of such polymeric materials seriously hampers drawing conclusions on structure–activity relationships to provide feedback on new vector designs. Furthermore, even for the most efficient systems, their clinical relevance would be seriously impaired due to the complex legal regulations that polydisperse entities have to meet.[15]

Multifunctional molecular vectors have emerged as a new generation of gene-delivery systems prone to chemical tailoring and systematic structural modification, thereby facilitating the elucidation of structure–activity relationships. A general approach is based on the use of preorganized macrocyclic scaffolds to achieve a precise alignment of functional elements, a strategy that has proven to be extremely useful over the years in the design of artificial receptors/ligands that emulate the supramolecular events occurring in living organisms. Thus, calixarene[16] and cyclodextrin-centered[17] starlike polycations have been synthesized and shown to exhibit promising pDNA delivery abilities. The tubular framework of CDs, which exhibit well-differentiated faces, can be further amended to bidirectional functionalization while keeping full homogeneity. The installation of segregated polycationic and hydrophobic domains (Figure 1)

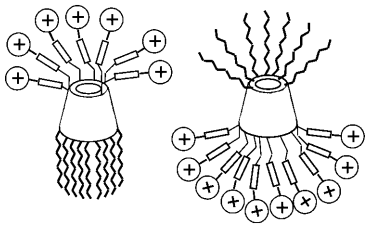


Figure 1. Schematic representation of skirt- (left) and jellyfish-type (right) polycationic amphiphilic CDs (paCDs). The circles and the rectangular boxes represent positively charged groups and spacer segments, respectively. The basket-shaped scaffold represents a cyclodextrin core (see Scheme 1).

could be exploited, then, to generate facial amphiphilicity,[18] thereby endowing the system with self-assembling properties and biomimetic[19] cell-membrane-crossing aptitudes.[20] We have now implemented this concept in the preparation of a library of monodisperse polycationic amphiphilic cyclodextrins (paCDs) that self-organize in the presence of pDNA to form stable paCD–pDNA complexes (CDplexes). A modular synthetic approach has been settled that allows systematic modification of the charge density, spacer length and nature, hydrophilic–hydrophobic balance, and overall architecture with high efficiency and relatively low synthetic cost. The effects of structural variations on the molecular construct in the CDplex stability and physicochemical properties, transfection efficacy, and cytotoxicity (BNL-CL2 and COS-7 cell lines) have been assessed and compared to that observed for commercial polycationic polymers (branched PEI, 25 kDa;[21] and linear JetPEI, 22 kDa).[22]

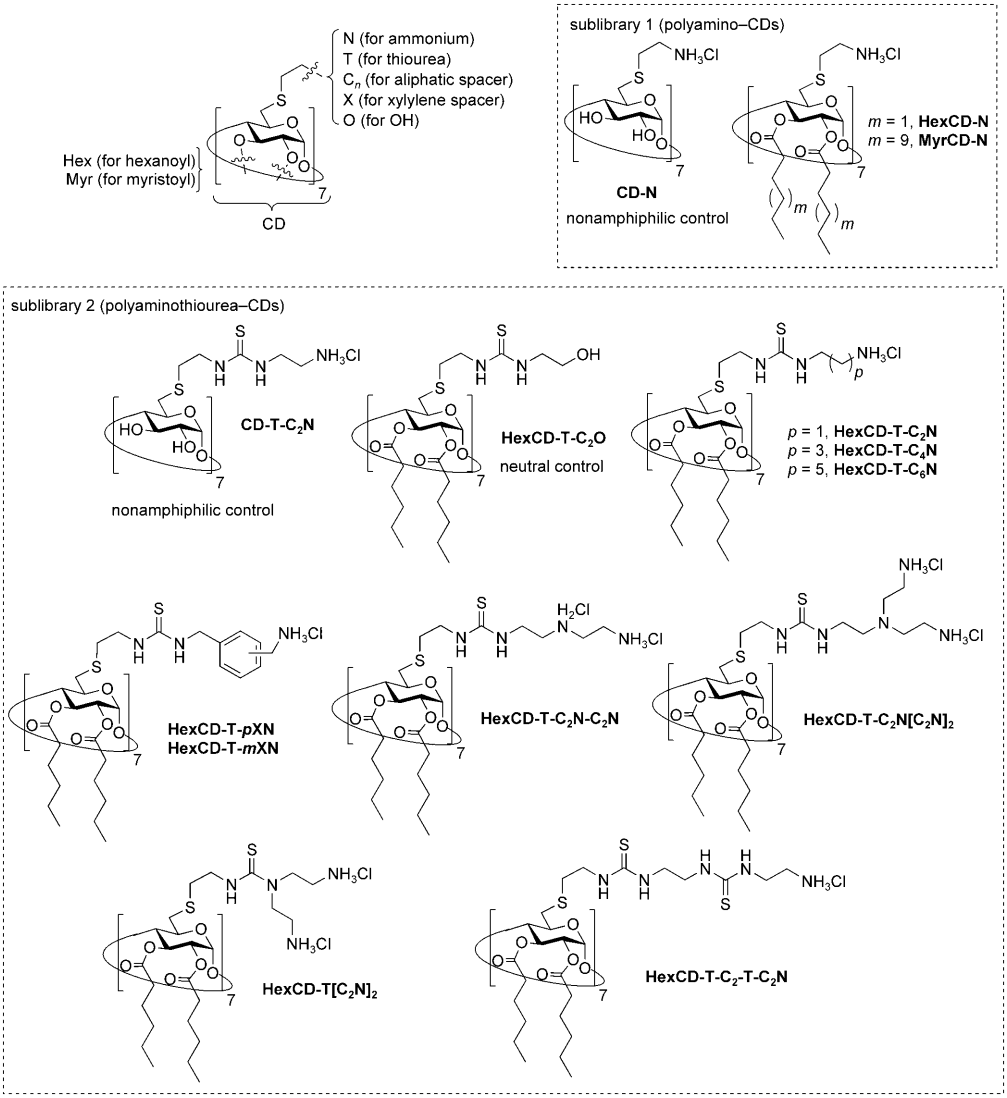
Results and Discussion

Design criteria and synthesis: The construction of multihead/multitail paCDs critically depends on the availability

of efficient face-selective functionalization methods of the CD scaffold. In principle, both possible relative orientations of the cationic groups and the hydrophobic elements, that is, the skirt or the jellyfish arrangements (Figure 1, left and right, respectively),[23] could be considered. Although a variety of synthetic strategies for regioselective manipulation of the primary hydroxyl groups (narrower rim) are at hand,[24] elaboration of the secondary CD hydroxyl groups (wider rim) has been scarcely explored.[25] Even relatively simple reactions, such as acylation, have been reported to lead to mixtures of undersubstituted or other undesired products when applied to this face,[26] which severely hampers purification of uniformly functionalized compounds that preserve the original C_n symmetry. Moreover, functionalization of the secondary hydroxyl groups generally requires prior protection of the more accessible primary hydroxyl groups, thus implying longer reaction sequences. We therefore focused on a skirt-type architecture for the purpose of this work. Since face-selective hydroxyl manipulation methodologies are common to the three commercially available CDs (α, β, and γCD), the most interesting representative from the commercial point of view, namely, cyclomaltoheptaose (βCD), was our chosen platform.

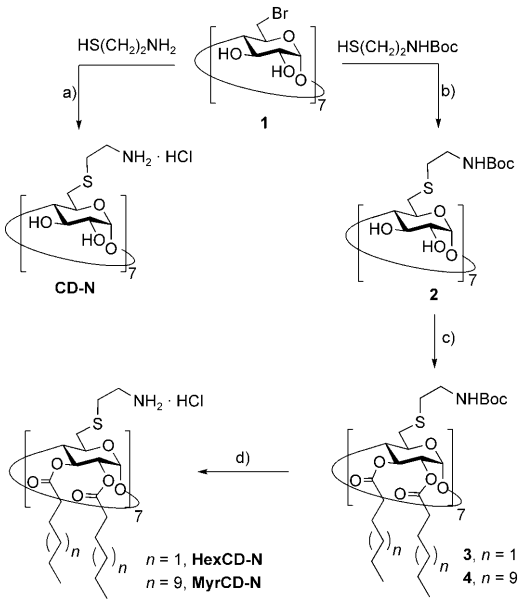
A semiconvergent, diversity-oriented strategy that allows modification of each of the constituent parts of the paCD construct in a modular manner has been disclosed. It involves: 1) replacement of the seven primary hydroxyl groups in βCD by *tert*-butoxycarbonyl (Boc)-protected cysteaminyl segments; 2) acylation of the fourteen secondary hydroxyl groups; 3) hydrolysis of the carbamate groups and; 4) further modification of the cationic heads by thiourea-forming reactions. In this way, two sublibraries were generated that consist of polyamino and polyaminothiourea βCD derivatives, respectively (Scheme 1).

The first transformation was accomplished in two steps from the commercially available cyclooligosaccharide by heptabromination with the *N*-bromosuccinimide (NBS)/triphenylphosphine (TPP) system (→**1**),[27] followed by cesium



Scheme 1. CD-scaffolded library members with indication of their structure-correlated notation.

carbonate promoted nucleophilic displacement of bromine by *N*-Boc-cysteamine (→**2**). We next found that acylation of the secondary hydroxyl groups could be achieved very efficiently by using fatty acid anhydrides in *N*,*N*-dimethylformamide (DMF) and *N*,*N*-dimethylaminopyridine (DMAP) as a non-nucleophilic base catalyst, which is significantly different from that reported when the corresponding acyl chlorides are employed.[20a] The tetradecahexanoate **3** and the tetradecamyristoate **4** were thus prepared. Subsequent acid-promoted hydrolysis of the *tert*-butyl carbamate afforded the corresponding heptacationic amphiphilic derivatives **HexCD-N** and **MyrCD-N** in a four-step process involving a single chromatographic purification on a multigram scale (Scheme 2).



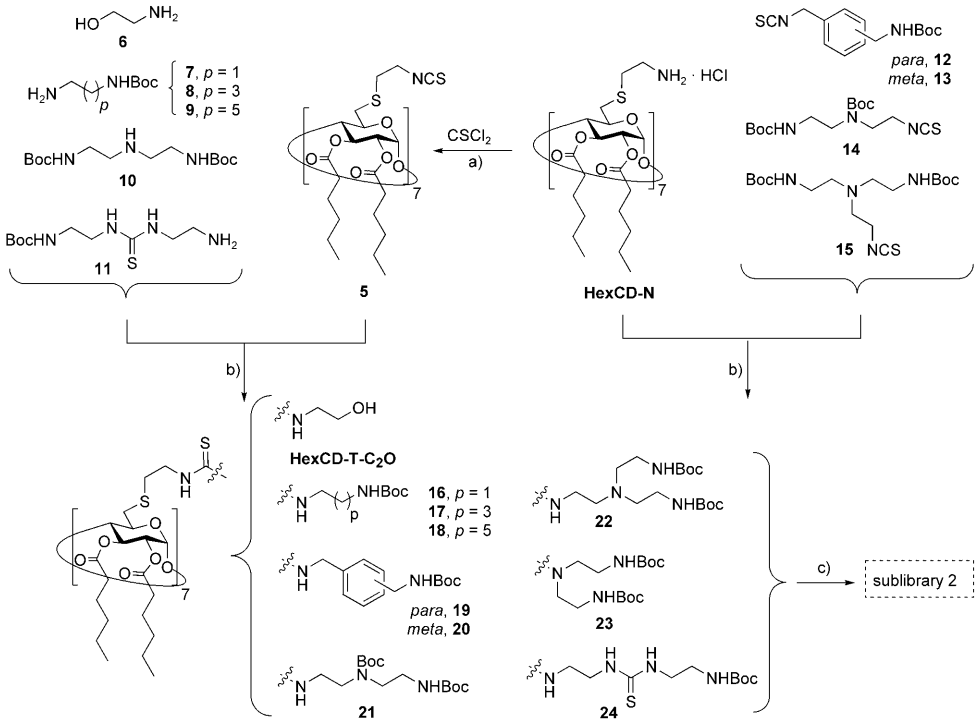
Scheme 2. Synthesis of polyamino cyclodextrins (sublibrary 1). Reagents and conditions: a) cysteamine hydrochloride, Et_3N, DMF, RT, 86% (ref. [28]); b) *N*-Boc cysteamine, Cs_2CO_3, DMF, 70°C, 66%; c) hexanoic ($n=1$) or myristic ($n=9$) anhydride, DMAP, DMF, 45 min, 76 and 67%, respectively; d) 1:1 TFA/CH_2Cl_2, RT, 2 h; then HCl, 99%.

The above compounds are the first representatives of the cysteaminyl–paCD series. The presence of the cysteamine segment in the structure is of importance since we already know that the accessibility of the amino groups on the CD core is much improved after insertion of the ethylene spacer between the nitrogen atom and the CD nucleus.[28] Such a reactivity enhancement should facilitate the formation of intermolecular salt bridges with phosphate anions in the pDNA skeleton. The length of the acyl chain was also expected to be a critical parameter. Actually, the compound bearing fourteen hexanoyl functionalities at the secondary face efficiently condensed the plasmid into nanometric CDplexes suitable for gene delivery (see below), whereas the myristoyl groups fully abolished the formation of self-assembled paCD:pDNA complexes, probably due to irreversible aggregation in aqueous media.

In view of the above considerations, the tetradecahexanoate derivative **HexCD-N** was chosen as the key precursor to enlarge the collection of amphiphilic CD architectures. The amine–isothiocyanate coupling reaction[29] was selected for this purpose for two main reasons. First, it generally proceeds in high yield and has already proven to be suitable for the preparation of homogeneous CD-scaffolded hyperbranched derivatives, thus avoiding overwhelming separations.[28,30] Second, the resulting adducts will incorporate a belt of hydrogen-bonding thiourea centers appropriately located to participate in cooperative binding to the phosphate groups in the plasmid chain. It is widely accepted that anion recognition and binding in biological systems does not rely on charge compensation exclusively, but rather depends on the presence of cooperative networks of hydrogen bonding and electrostatic interactions,[31] and this is true also for phosphates.[32] Actually, artificial oligosaccharide mimics incorporating thiourea connectors have been shown to bind phosphate anions even in aqueous media.[33]

In principle, poly(*N*,*N*′-disubstituted thiourea) derivatives can be synthesized by directly reacting the heptaamine with appropriately functionalized isothiocyanates. In the case of isothiocyanate partners susceptible of undergoing intramolecular reactions[34] or for the preparation of *N*,*N*′,*N*′-trisubstituted thiourea adducts, however, the corresponding heptaisothiocyanate **5**, prepared by isothiocyanation of **HexCD-N** with thiophosgene (Scheme 3), was instrumental. A set of amine (**6**–**11**) and isothiocyanate building blocks (**12**–**15**) was chosen to be able to investigate the effect that systematic variations in the distance between phosphate binding motifs (amine and thiourea functionalities), their relative disposition, the flexibility of the linkers, and the absence or the multiple presence of any of them might have in CD–DNA complex formation and delivery.

The individual Boc-protected sublibrary 2 members **16**–**24** were synthesized in parallel by coupling of **7**–**15** with the complementary heptaamine or heptaisothiocyanate CD reagent. In all cases the reaction proceeded to completion, as seen by TLC and MS of the reaction mixtures, in relatively short times. No side products arising from the self-condensation of the isothiocyanates, a source of side products when long reaction times or high temperatures are required,[34] were detected, and purification could be accomplished in all cases by normal flash chromatography (64–99% isolated yields). Trifluoroacetic acid (TFA)-catalyzed cleavage of the carbamate protecting groups in **16**–**24** and freeze-drying of the crude products from diluted HCl solutions afforded the target amphiphilic polyaminothiourea–CDs (sublibrary 2), as poly(hydrochloride) salts in virtually quantitative yield. NMR spectroscopy, ESIMS (see the Supplementary Information for a selection of spectra), and elemental microanalyses unequivocally demonstrated the structure and homogeneity of each library member. As an example, the ^{1}H NMR and ESIMS spectra of tetradecacationic CD **HexCD-T-[C_2N]$_2$**, with the typical single-spin system for a fully symmetric molecule and the peaks for the multiply charged molecular ions, respectively, are shown in Figure 2.



Scheme 3. Parallel synthesis of amphiphilic sublibrary 2 members. Reagents and conditions: a) $CaCO_3$, CH_2Cl_2/H_2O, RT, 2.5 h, 64%; b) Et_3N, CH_2Cl_2, RT, 16 h, 64–99%; c) 1:1 TFA/CH_2Cl_2, RT, 2 h, then HCl, 99%.

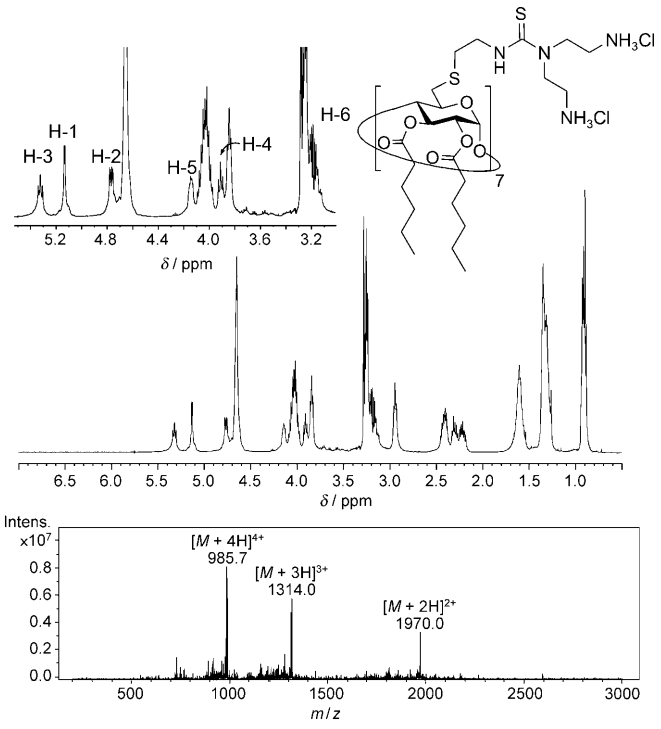
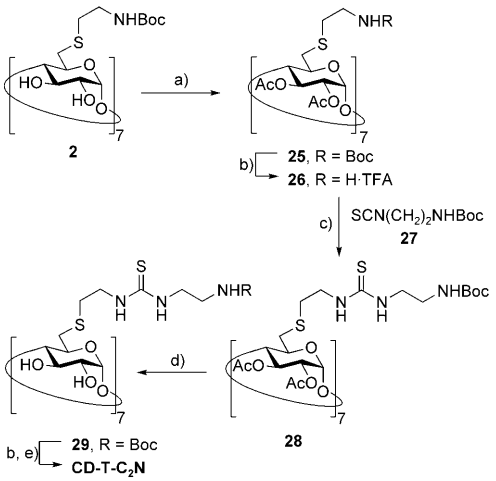


Figure 2. ^{1}H NMR spectra (500 MHz, 313 K, MeOD; top) and ESI mass spectra (bottom) of **HexCD-T[C_2N]$_2$**.

The amphiphilic hepta(hydroxyethylthiourea) derivative **HexCD-T-C_2O** was prepared as a neutral control compound to evaluate the relative contribution of hydrogen-bonding and electrostatic interactions in DNA complexation. In addition, a nonamphiphilic control, **CD-T-C_2N**, was also included in sublibrary 2 to estimate the effect of the presence of the hexanoyl chains. Its synthesis involved acetylation of the secondary hydroxyl groups in the *N*-(Boc)cysteaminyl–βCD derivative **2** (→**25**), followed by TFA-promoted carbamate hydrolysis, the coupling of the resulting heptaamine **26** with 2-(*tert*-butoxycarbonylamino)ethylisothiocyanate (**27**;[35] →**28**), deacetylation (→**29**), and finally, N-deprotection (Scheme 4).

pDNA complexation and CDplex characterization: Compounds in sublibraries 1 and 2 constitute a structurally diverse series of monodisperse paCDs very well suited for structure–gene transfection relationship studies. The **CD-N** and **CD-T-C_2N** derivatives, which lack the fatty acyl chains at the secondary face of the βCD core, were included in the polyamino and polyaminothiourea series, respectively, as nonamphiphilic control compounds. The capability of the various CDs shown in Scheme 1 to form stable CDplexes with pDNA (luciferase-encoding pTG11236) was determined at N/P ratios ranging from 5 to 30.[36,37] To avoid self-aggregation phenomena, the CD stock solutions were prepared in DMSO and further diluted with the pDNA solution in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (the final DMSO content never exceeded 1% v/v). These formulations were analyzed by agarose gel electrophoresis, with staining by the ethidium bromide intercalating agent, for assessing DNA complex formation and protection as well as DNA integrity. The paCD–pDNA



Scheme 4. Synthesis of the nonamphiphilic control **CD-T-C_2N**. Reagents and conditions: a) Ac_2O/pyridine, RT, 24 h, 81%; b) 1:1 TFA/CH_2Cl_2, RT, 2 h, 99%; c) Et_3N, CH_2Cl_2, RT, 24 h, 70%; d) NaOMe in MeOH, RT, 16 h, 99%; e) HCl, 99%.

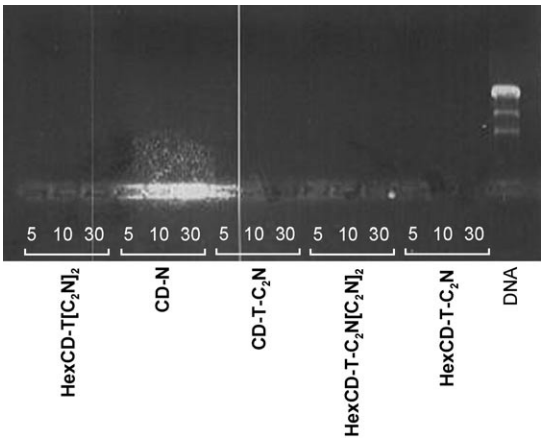


Figure 3. CD-mediated protection of pDNA from ethidium bromide intercalation at different N/P ratios (5, 10, and 30) for representative CDs (**HexCD-T[C_2N]$_2$**, **CD-N**, **CD-T-C_2N**, **HexCD-T-C_2N[C_2N]$_2$**, and **HexCD-T-C_2N[C_2N]$_2$**. Naked DNA is used for comparative purposes.

nanoparticles were further characterized by dynamic light scattering (DLS) for average hydrodynamic size (an important parameter to select potential candidates for in vivo uses),[38] mixed-mode measurement-phase analysis light scattering (M3-PALS) for zeta (ζ)-potential measurements, and transmission electron microscopy (TEM) for particle size and morphology.

Whereas the paCDs prepared in this study tend to form irregular aggregates in aqueous media, a templating effect resulting in compact, ordered, and stable nanoparticles was observed in the presence of the plasmid. The tetradecamyristoate **MyrCD-N** and the neutral derivative **HexCD-T-C_2O**, which were not dispersible in an aqueous environment, are two notable exceptions. The presence of a polycationic cluster and an appropriate hydrophilic/hydrophobic balance are, therefore, crucial features for triggering effective interactions with pDNA, thereby leading to compaction. Formation of the corresponding CDplexes was only achieved for the polycationic amphiphilic CDs, as shown by the agarose gel electrophoresis shift assay (Figure 3). Indeed, gel electrophoresis of the polycationic HexCD-based CDplexes indicated the absence of "free" mobile plasmid. Moreover, if pDNA is efficiently compacted and protected in the CDplexes, it becomes inaccessible to the ethidium bromide intercalating agent used as staining reagent for N/P ratios above 5, as demonstrated by the absence of fluorescent staining in the corresponding lanes. On the contrary, the gene in CDplexes formulated with the nonamphiphilic heptaamine derivative **CD-N** remained accessible to ethidium bromide in the whole range of N/P values from 5 to 30 (Figure 3, lanes 4–6). The presence of a thiourea segment in **CD-T-C_2N** results in a more efficient covering of the DNA surface, probably due to the interplay of electrostatic and hydrogen-bonding interactions. Thus, although the plasmid was still accessible to the intercalating agent for N/P 5 (Figure 3, lane 7), higher N/P values prevented ethidium-bromide staining (lanes 8 and 9). Intact recovery of pDNA upon SDS-induced CDplex dissociation demonstrated the reversibility of the complexation in all cases (data not shown).

The CD–pDNA nanoparticle hydrodynamic diameters and ζ potentials were next determined for different N/P ratios. Though all assayed polycationic CDs featured pDNA-complexing capabilities, the DLS results confirmed that the nonamphiphilic derivatives **CD-N** and **CD-T-C_2N** do not result in stable nanoparticles, further supporting the need of an amphiphilic architecture to promote the self-assembling process (Figure 4). Moreover, the **CD-N**:pDNA

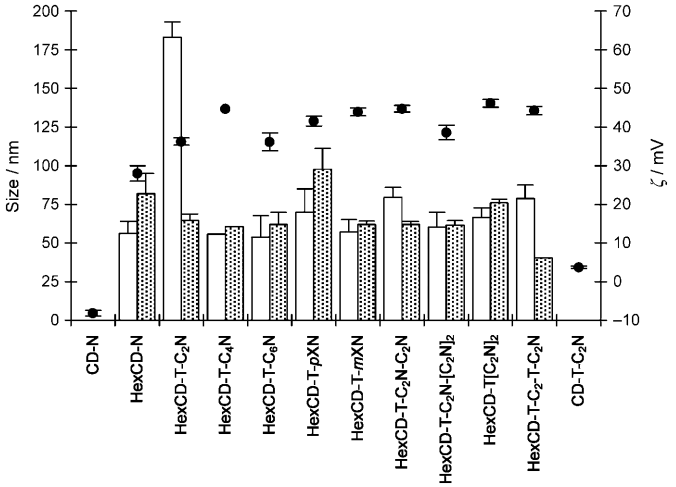


Figure 4. Size (hydrodynamic diameters, bars [nm]) and ζ potential (●, mV) of CDplexes determined by dynamic light scattering and M3-PALS analysis, respectively. Unfilled and filled bars correspond to nanoparticle size at N/P 5 and 10, respectively, while dots (●) represent the ζ potential at N/P 10. The measured size and ζ potential at N/P 30 did not differ significantly from those at N/P 10. Attempts to determine the size for CDplexes obtained from the nonamphiphilic control compounds **CD-N** and **CD-T-C_2N** were unsuccessful (nonreproducible and erratic measurements).

and **CD-T-C_2N**:pDNA CDplexes exhibited ζ-potential values close to neutrality even at a high N/P ratio, which is in agreement with the low pDNA-compacting potency. In sharp contrast, the paCD-based formulations displayed further positive ζ potentials in the range 20–50 mV, which increased upon increasing the N/P ratio and reached a plateau for N/P 10 (the ζ potentials measured for N/P 30 are identical to those at N/P 10; data not shown). These results suggest the prevalence of an ideal paCD–pDNA complex stoichiometry that occurs at an N/P ratio of approximately 10, above which the excess of paCD does not incorporate to the CDplex nanostructure.

Excepting the case of the **HexCD-T-C_2N**:pDNA complex at N/P 5, all the CDplexes prepared from paCDs exhibited rather small hydrodynamic diameters as compared with the polymer:pDNA complexes (polyplexes) obtained using branched poly(ethyleneimine) (bPEI, 25 kDa), one of the most efficient commercial gene-delivery systems[21] (50–80 nm vs. 150 nm). Additionally, quasi-monodisperse populations of cationic nanoparticles were observed in each individual experiment. Such remarkable behavior (low particle size and monodispersity) has only been reported for a monomolecular condensation process that occurs upon mixing DNA with dimerizable, polycationic detergents.[39,40a]

Transmission electron microscopy confirmed the small size and homogeneous distribution of CDplex formulations (Figure 5).[41] At high magnification, a snail-like ultrastructure was observed. These structures were probably made of alternating lamellar arrangements of paCDs and electron-dense densities corresponding to the pDNA molecule, a scenario that is reminiscent of the onionlike structure encountered in multimolecular siRNA:liposome complexes.[42] The rather small size and homogeneity of CDplexes makes them promising candidates for the development of systemic applications in vivo.[5,40] Thus, their gene-delivery properties would not suffer due to size-restricted diffusion and epithelial permeation.[43]

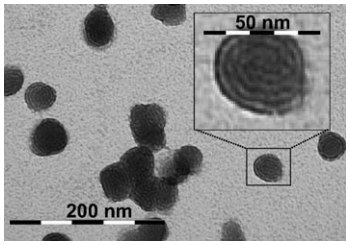


Figure 5. Transmission electron microscopy (TEM) images of **HexCD-T-[C_2N]$_2$**:pDNA CDplexes.

The effect of serum and of a saline medium on CDplex size and stability was next investigated to further assess their potential behavior under physiological conditions. A size increase from approximately 70–80 nm to 130–200 nm was observed when the **HexCD-T-C_2N**:pDNA or **HexCD-T-[C_2N]$_2$**:pDNA complexes obtained at N/P 10 were incubated in a 10% serum-containing medium. The increase in size was more pronounced when CDplexes were exposed to salt concentrations above 50 mM (8-fold and 3-fold for the **HexCD-T-C_2N**:pDNA and **HexCD-T[C_2N]$_2$**:pDNA complexes, respectively, at 250 mM NaCl), probably due to nanoparticle aggregation (Figure 6). In any case, the pDNA remained complexed and fully protected under these conditions, as highlighted by agarose gel electrophoresis (data not shown), which is significantly different from that reported for nonamphiphilic systems.[44]

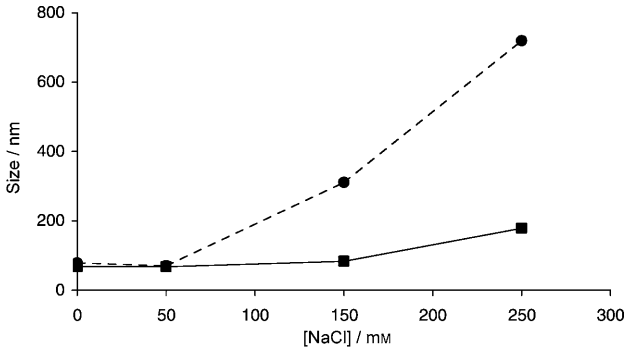


Figure 6. Salt-induced hydrodynamic diameter variation for **HexCD-T-C_2N**:pDNA (●) and **HexCD-T[C_2N]$_2$**:pDNA (■) CDplexes at N/P 7.

pDNA delivery and transfection: The transfection efficiency of the self-assembled paCD:pDNA complexes was evaluated using the luciferase-encoding reporter gene (pTG11236, pCMV-SV40-luciferase-SV40pA) in BNL-CL2, COS-7, and KB cells in HEPES buffer (20 mM, pH 7.4). CDplexes were formulated at different N/P ratios. Branched PEI (bPEI, 25 kDa) or linear JetPEI (22 kDa) and naked pDNA were used for comparative purposes (positive and negative controls, respectively). Stepwise structure–activity relationship (SAR) analyses were performed to assess the influence of each structural element on transfection efficiency.

Operating conditions were preliminarily optimized for CDplexes formulated with **HexCD-N** (sublibrary 1) or **HexCD-T-C_2N** (sublibrary 2). Maximum transfection efficiencies and cell viabilities were detected for N/P ratios 10 and 5, respectively. Higher N/P ratios not only did not improve performance, but also resulted in higher toxicity, probably due to aggregation of the free paCD. The CDplexes based on the nonamphiphilic polycationic analogues **CD-N** and **CD-T-C_2N** showed negligible gene-delivery properties (Figure 7), which was in sharp contrast to reported results for discrete nonamphiphilic CD polycations.[17] Most probably, the much poorer gene-condensing and -protecting capabilities featured by nonamphiphilic CDs together with their poor membrane-fusiogenic properties accounts for this inefficiency. Membrane-fusiogenic properties in lipoplexes are known to play a decisive role in favoring cell uptake and endosomal escape of DNA, by destabilizing anionic lipid-based biological membranes.[45]

SAR analysis demonstrated the superior abilities of amphiphilic polyaminothiourea CD derivatives (sublibrary 2)

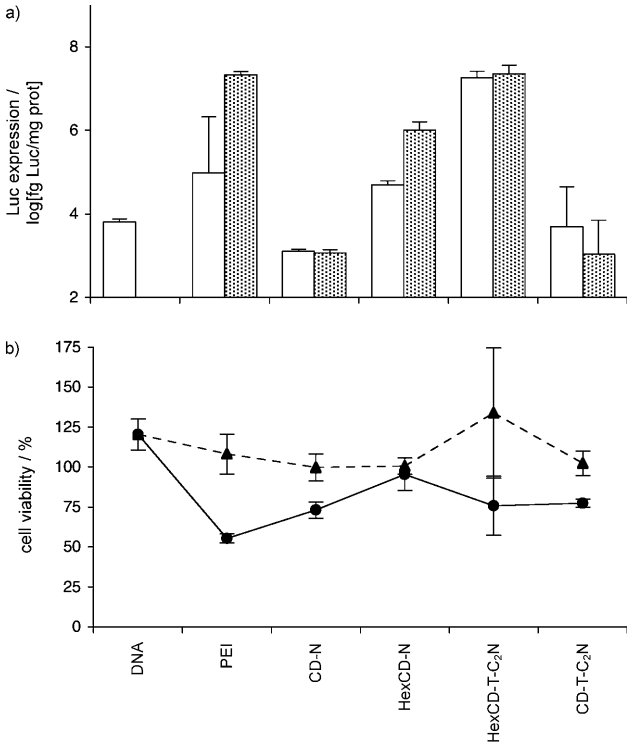


Figure 7. a) In vitro transfection efficiency and b) cell viability in BNL-CL2 cells of CDplexes formulated with **HexCD-N**, **HexCD-T-C_2N**, or the nonamphiphilic **CD-N** and **CD-T-C_2N** controls in comparison with data for naked DNA (=pTG11236) and bPEI (25 kDa)-based polyplexes at N/P 5 (unfilled bars and ▲) and 10 (filled bars and ●).

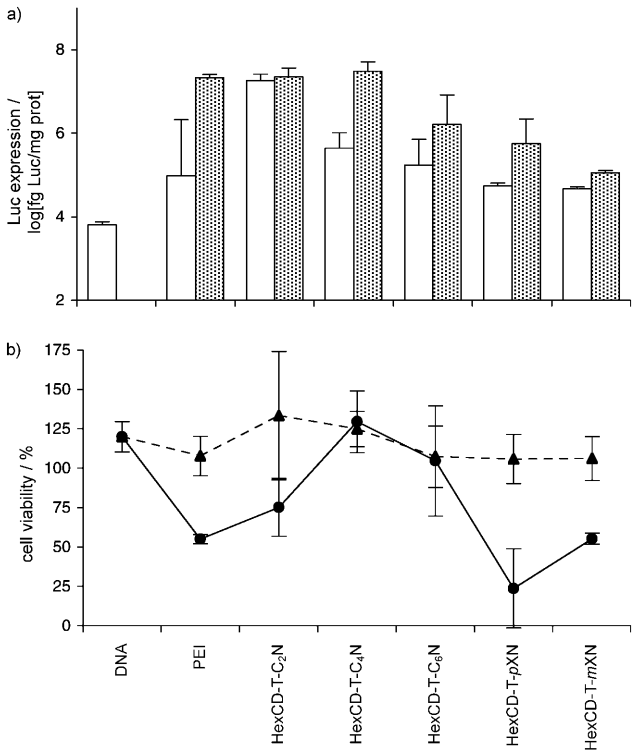


Figure 8. a) In vitro transfection efficiency and b) cell viability in BNL-CL2 cells of CDplexes formulated with heptaaminothioureido amphiphilic CDs differing in spacer length and flexibility versus naked DNA (=pTG11236) and bPEI (25 kDa)-based polyplexes at N/P 5 (unfilled bars and ▲) and 10 (filled bars and ●).

relative to polyamino CD derivatives (sublibrary 1) as transfection systems. Thus, the N/P 5 CDplexes made from **HexCD-T-C_2N** were over 100-fold and 10-fold more efficient than those obtained from **HexCD-N** at N/P 5 and 10, respectively (Figure 7). The fact that such an improvement in gene delivery and expression can be nanoengineered by inserting a rationally designed recognition element operating at the atomic level is remarkable. The performance of **HexCD-T-C_2N** at N/P 5 is actually similar to that shown by branched PEI at N/P 10, but featuring a far less toxic profile (cell viability $\geq$100%) than this commercial polycationic polymer (60% cell viability; Figure 7).

To assess the effect of different spacer arms between the thiourea moiety and the cationic centers, a further SAR evaluation within heptaammonium/heptathiourea sublibrary 2 members was conducted by comparing the transfection efficiency mediated by CDplexes formulated with **HexCD-T-C_nN** (n=2, 4, 6), or **HexCD-T-*p*** and ***m*XN**. From data in Figure 8, it can be inferred that luciferase expression decreases when increasing the distance between the amino and thioureido functions. At N/P 5, a 100-fold decrease in transfection efficiency was observed for **HexCD-T-C_6N** relative to **HexCD-T-C_2N**. At N/P 10, there is no decrease when going from **HexCD-T-C_2N** to **HexCD-T-C_4N**, but it was still above one order of magnitude from **HexCD-T-C_4N** to **HexCD-T-C_6N**. This observation highlights the benefits that a suitable preorganization of phosphate-recognition elements in the individual branches exert over the transfection efficiency of the corresponding supramolecular aggregates. That becomes more evident in the case of the compounds bearing aromatic spacers **HexCD-T-*p*XN** and **HexCD-T-*m*XN**, in which a concerted operation of thiourea and amino motifs in phosphate binding is probably impaired. Under the same assay conditions, luciferase expression for the corresponding CDplexes is reduced over 100-fold relative to **HexCD-T-C_2N**-based CDplexes. Moreover, toxicity turns to be much higher at N/P 10 (Figure 8).

The above results led us to identify the shorter and flexible ethylene segment as the optimal tether between thiourea and amine groups for gene delivery and expression mediated by CDplexes formulated with the paCDs. Keeping this element fixed, we next explored the effect of increasing the density of the amine and thiourea motifs. Deceptively, the higher-valent linear arrangements **HexCD-T-C_2N-C_2N**[46] and **HexCD-T-C_2-T-C_2N** resulted in lower transfection capabilities as compared with the heptaaminothiourea **HexCD-T-C_2N** in BNL-CL2 cells; this was in contrast with previous reports proposing a valency-dependent enhanced interaction with oligonucleotides for linear arrangements of both oligoethyleneimines[17a] and oligothioureas.[47] On the contrary, increasing the number of protonable amine groups in a dendritic display represented a significant improvement in vector design. In the case of **HexCD-T-C_2N[C_2N]$_2$**, though Figure 9 shows a performance similar to that of **HexCD-T-**

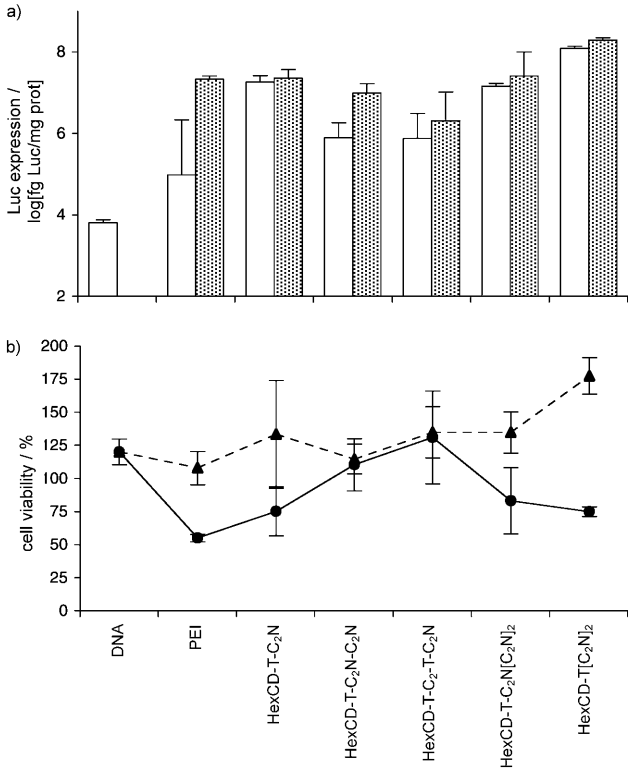


Figure 9. a) In vitro transfection efficiency and b) cell viability in BNL-CL2 cells of CDplexes formulated with amphiphilic polyaminothiourea CDs differing in the number and disposition of amine groups versus naked DNA (=pTG11236) and bPEI (25 kDa)-based polyplexes at N/P 5 (unfilled bars and ▲) and 10 (filled bars and ●).

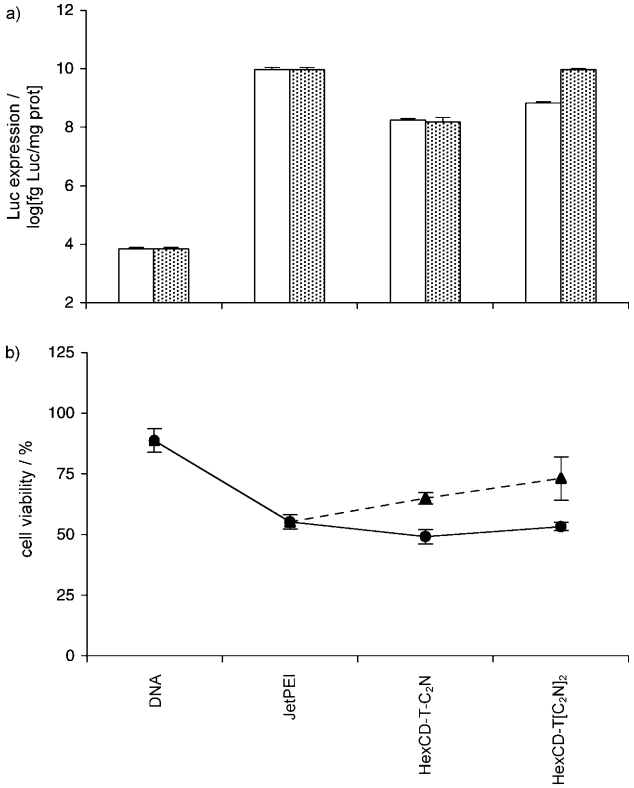


Figure 10. a) In vitro transfection efficiency and b) cell viability in COS-7 cells of **HexCD-T-C_2N**:DNA and **HexCD-T[C_2N]$_2$**:DNA CDplexes versus naked DNA (=pTG11236) and JetPEI-based polyplexes at N/P 5 (unfilled bars and ▲) and 10 (filled bars and ●).

C_2N, the fact that the structure of the former features 21 protonable groups (only 7 in **HexCD-T-C_2N**) implies that only one third of the paCD is required to achieve the same efficiency in molar terms. The better suited three-dimensional arrangement was that of **HexCD-T[C_2N]$_2$**. Despite lacking one of the hydrogen-bond donor centers at the thiourea moiety, **HexCD-T[C_2N]$_2$**:pDNA complexes at N/P 5 or 10 featured a 10-fold increase in luciferase production relative to the corresponding **HexCD-T-C_2N**:pDNA ones. In molar terms, that means that half the amount of **HexCD-T[C_2N]$_2$** (14 protonable amine groups) is sufficient to achieve even a higher transfection efficiency as compared with **HexCD-T-C_2N** (7 protonable amine groups). **HexCD-T[C_2N]$_2$**:pDNA complexes at N/P 5 are also one order of magnitude more efficient than bPEI-based polyplexes at its best N/P ratio of 10, while preserving a far less toxic profile (Figure 9). The transgene expression mediated by these CDplexes is over three orders of magnitude higher than that of bPEI-based N/P 5 polyplexes.

Preliminary results indicated that paCD-based CDplexes behave as broad-scope gene-delivery systems, with very similar architecture-dependent efficiency profiles in different cell lines, including COS-7 (Figure 10) and KB cells (data not shown). Paralleling results with BNL-CL2, multiplication of the phosphate-binding motifs in a dendritic manner (e.g., from **HexCD-T-C_2N** to **HexCD-T[C_2N]$_2$**) pushed transgene expression up to two orders of magnitude in COS-7 cells (Figure 10). At N/P 10, the transfection efficiency for **HexCD-T[C_2N]$_2$**-based CDplexes was similar to that observed for JetPEI-based polyplexes, but for a compound that is now perfectly homogeneous.

Often, many artificial gene vectors that exhibit successful gene delivery in vitro fail when trying to reproduce gene delivery under physiological conditions,[48] which is usually ascribed to complex instability in the presence of serum components. The serum saline stress (typically 150 mM) often promotes aggregation of cationic complexes, thereby leading to vascular blockage.[49] Additionally, cationic complexes readily bind with serum proteins, which hinders cellular uptake, promotes aggregation, and eventually leads to phagocytosis.[50] Shielding nanoparticles from salt and serum components is a way to achieve successful artificial gene vectors. In this context, the CDplex amphiphilic shell should contribute to protect nanoparticle fate. In fact, the above-mentioned nanoparticle stability assays have revealed the almost inertness of **HexCD-T[C_2N]$_2$**-based CDplexes toward saline stress and serum. To confirm this hypothesis, gene-delivery experiments in the absence and in the presence of serum were conducted. Luciferase expression in BNL-CL2 cells showed a decrease of three orders of magnitude with **HexCD-T-C_2N** in the presence of serum (Figure 11) relative to the assay in serum-free medium. Interestingly, in the case

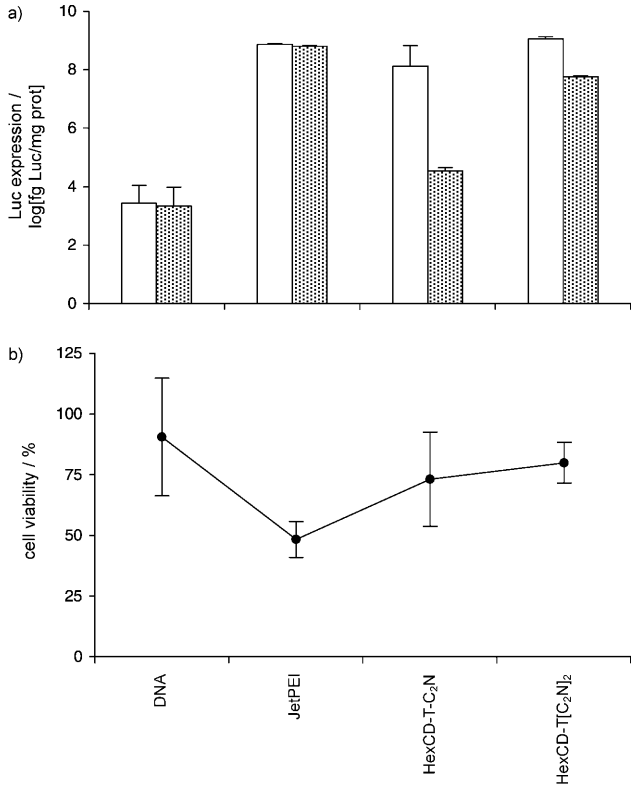


Figure 11. a) In vitro transfection efficiency and b) cell viability in BNL-CL2 of **HexCD-T-C_2N**:pDNA and **HexCD-T[C_2N]$_2$**:pDNA CDplexes at N/P 10 in the absence (unfilled bars) and in the presence (filled bars) of serum versus naked DNA and JetPEI-based polyplexes.

of **HexCD-T[C_2N]$_2$** CDplexes, the efficiency drop is very limited and remains close to that of JetPEI but with a lower cytotoxic profile.

Conclusion

In summary, we have implemented a straightforward design of polycationic CD-based facial amphiphiles as monodisperse molecular systems for efficient gene delivery and a diversity-oriented synthetic strategy suitable for SAR studies. The overall architecture of these paCDs can be finely tuned in terms of density of cationic groups, flexibility, and the presence of additional hydrogen-bonding functionalities while keeping a C_7-symmetric disposition. Control of the hydrophilic/hydrophobic balance between the CD primary and secondary faces proved crucial for pDNA complexation and nanoparticle (CDplex) formation. Most importantly, monodisperse populations of very small DNA nanoparticles (<100 nm), which is a prerequisite when in vivo systemic applications are sought, can be easily formulated from these paCDs.[51] Nanoparticle stability and transfection efficiency can be rationally modulated by judicious tailoring of the molecular topology. Remarkably, transfection efficiencies that surpass those of the polycationic polymers bPEI and JetPEI with lower cytotoxicity profiles have been achieved for BNL-CL2 and COS-7 cell lines by using paCDs that present a dendritic arrangement of cationic elements as in **HexCD-T[C_2N]$_2$**. The versatility of the synthetic scheme makes it potentially amenable to the selective installation of additional functional elements (e.g., for cell targeting, nuclear localization, monitoring cell uptake and fate) over the CD core, thus offering further opportunities for gene targeting that will merit investigation.[52]

It is worth stressing that the correlation of gene-vector chemical design and final transfection efficiency is only an overview of the whole process leading to transgene expression. Thus, comparable results on transfection efficiency encountered for dendritic paCD and PEI-based polyplexes in this study do not necessarily imply identical internalization mechanisms and intracellular trafficking routes. Investigation of the privileged pathways for cell uptake, escape of the intracellular endosomal compartments, dissociation of plasmid and carrier, nuclear translocation of the nucleic acids, and, finally, transcription of the transfected pDNA and protein expression is on the whole necessary to obtain the relevant information for optimization of the system. Research in that direction is currently in progress in our laboratories.[53]

Experimental Section

General methods: Reagents and solvents were purchased from commercial sources and used without further purification, with the following exceptions: dichloromethane was distilled under an Ar stream over CaH_2. Optical rotations were measured at 20 °C in 1 cm or 1 dm tubes using a Perkin–Elmer 141 MC polarimeter. IR spectra were recorded using an FTIR spectrometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 500 (125.7) and 300 (75.5) MHz. 2D COSY, 1D TOCSY, and HMQC experiments were used to assist with the NMR spectroscopy assignments. NMR spectra and a guide to the notation used for the assignments can be found in the Supporting Information. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with Kieselgel 60 F_{245} (E. Merck), with visualization by UV light and by charring with 10% H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was carried out on silica gel 60 (E. Merck, 230–400 mesh). Electrospray mass spectra (ESIMS) were obtained using a Bruker Esquire6000 instrument. Elemental analyses were performed at the Instituto de Investigaciones Químicas (Sevilla, Spain).

Preparation of complexes composed of CD 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 quantities of compound used were calculated according to the desired DNA concentration of 0.1 mg mL^{-1} (303 μM phosphate), the N/P ratio, the molar weight, and the number of protonable nitrogen atoms in the selected CD derivative or cationic polymer (bPEI, 25 kDa or JetPEI). Experiments were performed for N/P 5, 10, 30, and 50. For the preparation of the DNA complexes from CD derivatives and PEI, DNA was diluted in HEPES (20 mM, pH 7.4) to a final concentration of 303 μM, then the desired amount of CD derivative was added from 10 or 20 mM stock solution (DMSO) and bPEI (25 kDa) 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 303 μM, then the desired amount of JetPEI was added from a 7.5 mM water solution. The preparation was vortexed for 2 h and used for characterization or transfection experiments.

Measurement of the size of the complexes by dynamic light scattering (DLS) and of the zeta potential: The average sizes of the CDplexes were

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°; $\lambda = 633$ 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. Zeta-potentials measurements on the CDplexes were made using the same apparatus with "mixed-mode measurement" phase analysis light scattering.

"**Mixed-mode measurement" phase analysis light scattering (M3-PALS)**: M3 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 instruments was checked with either a 90 nm monodisperse latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for zeta 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× tris(hydroxymethyl)aminomethane (Tris)/acetate/ethylenediaminetetraacetic acid (EDTA) (TAE) buffer and stained by spreading an EtBr (Sigma) solution in TAE buffer (20 μL ethidium bromide of a 10 mg mL^{-1} solution in 200 mL TAE). The DNA was then visualized after photographing using an UV transilluminator. The plasmid integrity in each sample was confirmed by electrophoresis after decomplexation with sodium dodecyl sulfate (SDS, 8%).

Statistical analysis: Statistical tests were performed with STATGRAPHICS Plus 5.0 software.[54] Analysis of variance (Anova) was run on the logarithmic transformation of transfection levels (log 10(fg luciferase per mg protein)) and on the cell viability to fit normal distributions of the data. Two factors, that is, the nature of the complexing agent (CD derivative and PEI) and the N/P ratio, were analyzed as the source of the variation of logarithmic transformation of the transfection levels and of cell variability percentages using a multiple comparison procedure. Tukey's honestly significant difference (HSD) method was used to discriminate among the means of cell viability percentages and the logarithmic transformation of luciferase expression levels.

In vitro transfection: Twenty-four hours before transfection, BNL-CL2 or 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), 10 mg mL^{-1} gentamycin for BNL-CL2 cells, or 100 units per mg penicillin and 100 μg mL^{-1} streptomycin for COS-7 cells, in a wet (37 °C) and 5% CO_2/95% air atmosphere. The above-described CD:pDNA (=pTG11236) complexes and PEI:pDNA polyplexes were diluted to 100 μL in DMEM or 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 μL) 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).

Transmission electron microscopy (TEM): Formvar-carbon-coated grids previously made hydrophilic by glow discharge were placed on top of small drops of the CDplex samples (HEPES 20 mM, pH 7.4, DNA 303 μM phosphate) prepared as described above. After 1–3 min of contact, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grids were then dried and observed using a Philips CM12 electron microscope working under standard conditions. All these experiments were reproduced twice on each formulation.

Synthesis: The starting materials, heptakis(6-bromo-6-deoxy)cyclomaltoheptaose (**1**),[27] heptakis[6-(2-aminoethylthio)-6-deoxy]cyclomaltoheptaose (**CD-N**),[28] 4- and 3-(*tert*-butoxycarbonylaminomethyl)benzyl isothiocyanate (**12**, **13**),[55] and 2-(*tert*-butoxycarbonylamino)ethyl isothiocyanate (**27**)[35] were prepared as described previously.

Compound 2: *tert*-Butyl *N*-(2-mercaptoethyl)carbamate (5.3 mmol, 1.4 equiv) was added to a suspension of heptakis(6-bromo-6-deoxy)cyclomaltoheptaose[27] (**1**, 0.84 g, 0.53 mmol) and Cs_2CO_3 (1.71 g, 5.25 mmol) in dry DMF (10 mL). The suspension was heated, under an Ar atmosphere, at 70 °C for 48 h. The reaction mixture was cooled to RT, poured into ice water (30 mL), and stirred overnight. The resulting solid was filtered and washed with a large volume of water, and then with a small amount of cold Et_2O. The residue was purified by flash chromatography (40:10:1→30:10:1 CH_2Cl_2/MeOH/water). Yield: 787 mg (66%); $R_f = 0.60$ (40:10:1 CH_2Cl_2/MeOH/water); $[\alpha]_D = +79.7$ ($c = 0.8$ in MeOH); ^{1}H NMR (500 MHz, MeOD, 323 K): $\delta = 4.95$ (d, $J_{1,2} = 3.5$ Hz, 7H; H-1), 4.00 (m, 7H; H-5), 3.86 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 7H; H-3), 3.48 (dd, 7H; H-2), 3.26 (t, ^3J(H,H) = 7.5 Hz, 21H; H-4, CH_2N_{Cyst}), 3.20 (m, 7H; H-6a), 2.91 (m, 7H; H-6b), 2.77 (m, 14H; CH_2S_{Cyst}), 1.45 ppm (brs, 63H; CMe_3); ^{13}C NMR (125.7 MHz, MeOD, 323 K): $\delta = 156.1$ (CO), 104.1 (C-1), 86.5 (C-4), 80.3 (C_q), 74.6 (C-3), 74.4 (C-2), 73.3 (C-5), 41.8 (CH_2N_{Cyst}), 34.9 (CH_2S_{Cyst}), 34.5 (C-6), 29.1 ppm (CMe_3); ESIMS: *m*/*z*: 1147.4 $[M+2Na]^{2+}$, 2271.8 $[M+Na]^+$; elemental analysis calcd (%) for $C_{91}H_{161}N_7O_{42}S_7$: C 48.58, H 7.21, N 4.36; found: C 48.44, H 6.80, N 4.27.

Compound 3: DMAP (4.56 g, 37.3 mmol, 3 equiv) and hexanoic anhydride (12 mL, 49.8 mmol, 4.0 equiv) were added to a solution of **2** (2.0 g, 0.89 mmol) in dry DMF (10 mL) under Ar. The mixture was heated at 70 °C for 4–5 h. Then, MeOH (10 mL) was added and the mixture was further stirred at 70 °C for 3 h. The mixture was poured into ice water (50 mL) and extracted with CH_2Cl_2 (4×50 mL). The organic phase was washed with diluted H_2SO_4 (2×50 mL), water, and cold saturated aqueous $NaHCO_3$ (4×50 mL), dried (Na_2SO_4), concentrated and purified by flash chromatography (1:3 EtOAc/petroleum ether). Yield: 2.44 g (76%); $R_f = 0.45$ (1:2 EtOAc/petroleum ether); $[\alpha]_D = +84.1$ ($c = 0.9$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$, 323 K): $\delta = 5.45$ (m, 7H; NH), 5.25 (t, $J_{2,3} = J_{3,4} = 9.5$ Hz, 7H; H-3), 5.08 (d, $J_{1,2} = 3.5$ Hz, 7H; H-1), 4.76 (dd, 7H; H-2), 4.12 (ddd, $J_{4,5} = 9.5$ Hz, $J_{5,6b} = 5.5$ Hz, $J_{5,6a} = 2.5$ Hz, 7H; H-5), 3.77 (t, 7H; H-4), 3.30, 3.29 (2t, ^3J(H,H) = 6.5 Hz, 14H; CH_2N_{Cyst}), 3.10 (dd, $J_{6a,6b} = 14.0$ Hz, 7H; H-6a), 3.02 (dd, 7H; H-6b), 2.74, 2.72 (2dt, ^2J(H,H) = 13.5 Hz, ^3J(H,H) = 7.0 Hz, 14H; CH_2S_{Cyst}), 2.38–2.11 (m, 28H; CH_2CO), 1.57 (m, 28H; CH_2CH_2CO), 1.42 (brs, 63H; CMe_3), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.89, 0.87 ppm (2t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 323 K): $\delta = 173.2$, 171.6 (CO ester), 155.9 (CO carbamate), 96.7 (C-1), 79.2 (CMe_3), 78.8 (C-4), 71.4 (C-5), 70.6 (C-3), 70.3 (C-2), 40.5 (CH_2N_{Cyst}), 34.0 (C-6, CH_2CO), 33.9 (CH_2S_{Cyst}), 33.8 (CH_2CO), 31.5, 31.3 ($CH_3CH_2CH_2$), 28.5 (CMe_3), 24.4, 24.3 (CH_2CH_2CO), 22.3, 22.2 (CH_3CH_2), 13.8 ppm (CH_3); ESIMS: *m*/*z*: 1833.9 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{175}H_{301}N_7O_{56}S_7$: C 58.00, H 8.37, N 2.71; found: C 57.79, H 8.19, N 2.50.

Compound 4: A mixture of **2** (0.2 g, 89 μmol), DMAP (0.46 g, 3.73 mmol, 3 equiv), and myristic anhydride (2.18 g, 4.97 mmol, 4.0 equiv) was dissolved in dry DMF (15 mL) at 0 °C under an Ar atmosphere. The resulting suspension was stirred at RT for 48 h, and the solvent was evaporated under diminished pressure. The resulting residue was refluxed with CH_2Cl_2/MeOH (5:95, 100 mL) for 1 h, decanted, and the residue was purified by flash chromatography (1:2 EtOAc/petroleum ether). Yield: 0.32 g (67%); $R_f = 0.56$ (1:2 EtOAc/petroleum ether); $[\alpha]_D = +49.2$ ($c = 1.0$ in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): $\delta = 5.38$ (brs, 7H; NH), 5.26 (t, $J_{2,3} = J_{3,4} = 8.9$ Hz, 7H; H-3), 5.08 (d, $J_{1,2} = 3.8$ Hz, 7H; H-1), 4.74 (dd, 7H; H-2), 4.13 (m, 7H; H-5), 3.76 (t, $J_{4,5} = 8.7$ Hz, 7H; H-4), 3.30 (brd, ^3J(H,H) = 6.1 Hz, 14H; CH_2N_{Cyst}), 3.10 (d, $J_{6a,6b} = 12.5$ Hz, 7H; H-6a), 3.01 (brd, 7H; H-6b), 2.72, 2.70 (2dt, ^2J(H,H) = 13.5 Hz, ^3J-

(H,H) = 7.0 Hz, 14H; CH_2S_{Cyst}), 2.38–2.11 (m, 28H; CH_2CO), 1.53 (m, 28H; CH_2CH_2CO), 1.43 (brs, 63H; CMe_3), 1.25 (m, 280H; CH_2), 0.88 ppm (2t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 173.3, 171.6 (CO ester), 155.9 (CO carbamate), 96.6 (C-1), 79.2 (CMe_3), 78.7 (C-4), 71.3 (C-5), 70.5 (C-3, C-2), 40.4 (CH_2N_{Cyst}), 34.2 (CH_2CO), 33.9 (C-6, CH_2S_{Cyst}), 32.0 ($CH_2CH_2CH_3$), 29.8–29.3 (CH_2), 28.5 (CMe_3), 24.8, 24.7 (CH_2CH_2CO), 22.6 (CH_2CH_3), 14.0 ppm (CH_3); ESIMS: m/z: 2619.5 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{287}H_{5251}N_7O_{56}S_7$: C 66.36, H 10.19, N 1.89; found: C 66.01, H 9.62, N 1.65.

Compound HexCD-N: Treatment of carbamate **3** (0.71 g, 0.2 mmol) with 1:1 TFA/CH_2Cl_2 (20 mL) at RT for 2 h, followed by evaporation of the solvents and freeze-drying from a diluted HCl solution, gave **HexCD-N** in virtually quantitative yield (0.6 g). $[\alpha]_D$ = +72.6 (c = 1.0 in MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 5.37 (t, $J_{2,3}=J_{3,4}$ = 9.5 Hz, 7H; H-3), 5.16 (d, $J_{1,2}$ = 3.5 Hz, 7H; H-1), 4.79 (dd, 7H; H-2), 4.07 (brt, $J_{5,6a}=J_{5,6b}$ = 6.5 Hz, 7H; H-5), 3.90 (t, $J_{4,5}$ = 9.0 Hz, 7H; H-4), 3.22 (d, $J_{6a,6b}$ = 13.0 Hz, 7H; H-6a), 3.21 (brt, ^3J(H,H) = 7.0 Hz, 14H; CH_2N_{Cyst}), 3.04 (dd, 7H; H-6b), 3.02, 2.94 (2dt, ^2J(H,H) = 14.5 Hz, ^3J(H,H) = 7.5 Hz, 14H; CH_2S_{Cyst}), 2.47–2.21 (m, 28H; CH_2CO), 1.62 (m, 28H; CH_2CH_2CO), 1.33 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.92, 0.91 ppm (2t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD): δ = 174.4, 174.3 (CO), 98.2 (C-1), 80.0 (C-4), 74.0 (C-5), 71.8 (C-2), 71.3 (C-3), 40.2 (CH_2N_{Cyst}), 35.2, 35.0 (CH_2CO), 34.4 (C-6), 32.6, 32.5 ($CH_3CH_2CH_2$), 31.7 (CH_2S_{Cyst}), 25.6, 25.5 (CH_2CH_2CO), 23.6, 23.5 (CH_3CH_2), 14.4, 14.3 ppm (CH_3); ESIMS: m/z: 1462.1 $[M+2H]^{2+}$, 974.8 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{140}H_{252}Cl_7N_7O_{42}S_7$: C 52.91, H 7.99, N 3.09: found: C 52.66, H 7.84, N 2.98.

Compound MyrCD-N: Treatment of the heptacarbamate **4** (112 mg, 21.6 μmol) with 1:1 TFA/CH_2Cl_2 (2 mL) at RT for 2 h, followed by evaporation of the solvent and freeze-drying from a diluted HCl solution, gave **MyrCD-N** in virtually quantitative yield (102 mg). $[\alpha]_D$ = + 43.3 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, 9:1 MeOD/$CDCl_3$, 323 K): δ = 5.36 (t, $J_{2,3}=J_{3,4}$ = 9.0 Hz, 7H; H-3), 5.12 (d, $J_{1,2}$ = 3.5 Hz, 7H; H-1), 4.77 (dd, 7H; H-2), 4.12 (m, 7H; H-5), 3.85 (t, $J_{4,5}$ = 9.0 Hz, 7H; H-4), 3.20 (m, 21H; H-6a, CH_2N_{Cyst}), 3.05 (m, 7H; H-6b), 2.99, 2.95 (2dt, ^2J(H,H) = 14.0 Hz, ^3J(H,H) = 7.0 Hz, 14H; CH_2S_{Cyst}), 2.45–2.18 (m, 28H; CH_2CO), 1.59 (m, 28H; CH_2CH_2CO), 1.30 (m, 280H; CH_2), 0.88 ppm (t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, 9:1 MeOD-$CDCl_3$): δ = 174.3, 173.5 (CO), 98.2 (C-1), 80.3 (C-4), 73.6 (C-5), 71.9 (C-2), 71.4 (C-3), 40.0 (CH_2N_{Cyst}), 35.0 (CH_2CO), 34.3 (C-6), 33.2 ($CH_2CH_2CH_3$), 31.7 (CH_2S_{Cyst}), 31.3–30.1 (CH_2), 26.0, 25.9 (CH_2CH_2CO), 23.9 (CH_2CH_3), 14.8 ppm (CH_3); ESIMS: m/z: 4491.6 $[M+H]^+$; elemental analysis calcd (%) for $C_{252}H_{476}Cl_7N_7O_{42}S_7$: C 63.73, H 10.10, N 2.06; found: C 63.55, H 9.84, N 1.95.

Compound 5: $CaCO_3$ (528 mg, 5.28 mmol, 4 equiv) and $CSCl_2$ (207 μL, 2.64 mmol, 2 equiv) were added to a solution of the heptaamine **HexCD-N** (600 mg, 189 μmol) in a mixture of acetone (2.4 mL) and water (3.6 mL). The reaction mixture was stirred for 2.5 h and then concentrated. The residue was dissolved in CH_2Cl_2 (6 mL) and washed with saturated aqueous $NaHCO_3$ (6 mL). The organic phase was decanted, dried (Na_2SO_4), and concentrated. The residue was subjected to flash chromatography (1:4→1:3 EtOAc/petroleum ether). Yield: 390 mg (64%); R_f = 0.34 (1:3 EtOAc/petroleum ether); $[\alpha]_D$ = +118.8 (c = 1.0 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.26 (t, $J_{2,3}=J_{3,4}$ = 9.5 Hz, 7H; H-3), 5.05 (d, $J_{1,2}$ = 4.0 Hz, 7H; H-1), 4.77 (dd, 7H; H-2), 4.13 (ddd, $J_{4,5}$ = 8.5 Hz, $J_{5,6b}$ = 5.5 Hz, $J_{5,6a}$ = 2.5 Hz, 7H; H-5), 3.81 (t, 7H; H-4), 3.77 (t, ^3J(H,H) = 7.0 Hz, 14H; CH_2N_{Cyst}), 3.16 (dd, $J_{6a,6b}$ = 14.5 Hz, 7H; H-6a), 3.04 (dd, 7H; H-6b), 3.00, 2.90 (2dt, ^2J(H,H) = 13.5 Hz, ^3J(H,H) = 6.5 Hz, 14H; CH_2S_{Cyst}), 2.38–2.11 (m, 28H; CH_2CO), 1.55 (m, 28H; CH_2CH_2CO), 1.27 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.88, 0.86 ppm (2t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$): δ = 173.4, 171.7 (CO), 132.2 (NCS), 96.7 (C-1), 78.5 (C-4), 71.6 (C-5), 70.2 (C-2, C-3), 45.6 (CH_2N_{Cyst}), 34.1 (C-6, CH_2S_{Cyst}), 34.0, 33.8 (CH_2CO), 31.4, 31.3 ($CH_3CH_2CH_2$), 24.4, 24.3 (CH_2CH_2CO), 22.4, 22.3 (CH_3CH_2), 13.9 ppm (CH_3); ESIMS: m/z: 1628.0 $[M+2H]^{2+}$, 722.6 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{147}H_{231}N_7O_{42}S_{14}$: C 54.88, H 7.24, N 3.05; found: C 54.98, H 7.37, N 3.02.

Compound HexCD-T-O: A solution of **5** (112 mg, 35 μmol) in dry CH_2Cl_2 (2 mL) was added dropwise to a solution of ethanolamine (**6**, 0.37 mmol, 22.2 μL, 1.5 equiv) in dry CH_2Cl_2 (1 mL). The mixture was stirred at RT for 16 h and the reaction mixture was quenched with water (10 mL) and extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were dried (Na_2SO_4), concentrated, and purified by flash chromatography (9:1→6:1 CH_2Cl_2/MeOH). Yield: 82 mg (64%); R_f = 0.34 (6:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +80.9 (c = 1.0 in MeOH); ^{1}H NMR (500 MHz, MeOD, 313 K): δ = 5.32 (t, $J_{2,3}=J_{3,4}$ = 8.6 Hz, 7H; H-3), 5.14 (d, $J_{1,2}$ = 3.6 Hz, 7H; H-1), 4.80 (dd, 7H; H-2), 4.18 (m, 7H; H-5), 3.90 (t, $J_{4,5}$ = 8.5 Hz, 7H; H-4), 3.74 (m, 14H; CH_2N_{cyst}), 3.68 (t, ^3J(H,H) = 5.4 Hz, 14H; CH_2OH), 3.58 (brs, 14H; CH_2NH), 3.26 (m, 7H; H-6a), 3.16 (dd, $J_{6a,6b}$ = 14.3 Hz, $J_{5,6b}$ = 5.6 Hz, 7H; H-6b), 2.93, 2.87 (2dt, ^2J(H,H) = 13.3 Hz, ^3J(H,H) = 6.8 Hz, 14H; CH_2S_{cyst}), 2.45–2.20 (m, 28H; CH_2CO), 1.62 (m, 28H; CH_2CH_2CO), 1.32 (m, 56H; CH_2CH_3, $CH_2CH_2CH_3$), 0.93, 0.90 ppm (2t, ^3J(H,H) = 7.2 Hz, ^3J(H,H) = 7.1 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 313 K): δ = 183.8 (CS), 174.7, 173.6 (CO), 98.2 (C-1), 80.0 (C-4), 73.2 (C-5), 72.0 (C-3), 71.7 (C-2), 61.8 (CH_2OH), 47.7 (CH_2NH), 45.3 (CH_2N_{cyst}), 35.2 (CH_2CO), 35.0 (C-6), 34.1 (CH_2S_{cyst}), 32.6, 32.5 ($CH_2CH_2CH_3$), 25.6 (CH_2CH_2CO), 23.5 (CH_2CH_3), 14.5, 14.3 ppm (CH_3); ESIMS: m/z: 1844.6 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{161}H_{280}N_{14}O_{49}S_{14}$: C 53.05, H 7.74, N 5.38; found: C 52.67, H 7.61, N 5.19.

Compound 16: A solution of **5** (114 mg, 35 μmol) in dry CH_2Cl_2 (2 mL) was added dropwise to a solution of *N-tert*-butoxycarbonylethylendiamine (**7**, 0.37 mmol, 59 mg, 1.5 equiv) in dry CH_2Cl_2 (1 mL). The mixture was stirred at RT for 20 h and the reaction mixture was quenched with water (10 mL) and extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were dried (Na_2SO_4), concentrated and purified by flash chromatography (20:1 CH_2Cl_2/MeOH). Yield: 129 mg (85%); R_f = 0.27 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +78.4 (c = 1.05 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.19 (brs, 1H; NH), 7.00 (brs, 1H; NH), 5.46 (brs, 1H; NH), 5.25 (t, $J_{2,3}=J_{3,4}$ = 8.9 Hz, 7H; H-3), 5.08 (d, $J_{1,2}$ = 3.7 Hz, 7H; H-1), 4.76 (dd, 7H; H-2), 4.15 (m, 7H; H-5), 3.75 (t, $J_{4,5}$ = 8.7 Hz, 7H; H-4), 3.72 (m, 14H; CH_2N_{Cyst}), 3.58 (m, 14H; CH_2NH), 3.29 (m, 14H; CH_2NHBoc), 3.20 (brd, $J_{6a,6b}$ = 13.4 Hz, 7H; H-6a), 3.04 (dd, $J_{5,6b}$ = 4.8 Hz, 7H; H-6b), 2.93, 2.81 (2dt, ^2J(H,H) = 13.3 Hz, ^3J(H,H) = 6.6 Hz, 14H; CH_2S_{Cyst}), 2.38–2.11 (m, 28H; CH_2CO), 1.56 (m, 28H; CH_2CH_2CO), 1.42 (s, 63H; CMe_3), 1.24 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.90, 0.87 ppm (2t, ^3J(H,H) = 7.3 Hz, ^3J(H,H) = 6.9 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 182.0 (CS), 173.4, 171.6 (CO ester), 156.9 (CO carbamate), 96.7 (C-1), 79.8 (CMe_3), 79.0 (C-4), 71.5 (C-5), 70.5 (C-3), 70.4 (C-2), 44.6 (CH_2NH), 43.8 (CH_2N_{Cyst}), 40.0 (CH_2NHBoc), 34.0 (CH_2CO), 33.8 (C-6), 32.9 (CH_2S_{Cyst}), 31.4, 31.2 ($CH_3CH_2CH_2$), 28.5 (CMe_3), 24.4, 24.3 (CH_2CH_2CO), 22.3 (CH_3CH_2), 13.8 ppm (CH_3); ESIMS: m/z: 2191.2 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{196}H_{343}N_{21}O_{56}S_{14}$: C 54.26, H 7.97, N 6.78; found: C 54.10, H 7.84, N 6.67.

Alternatively, **16** was prepared from **HexCD-N** as follows: A solution of 2-(*tert*-butoxycarbonylamino)ethylisothiocyanate[35] (**27**, 39 mg, 192 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL) was added to a solution of **HexCD-N** (80 mg, 25 μmol) and Et_3N (26 μL, 192 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 98 mg (90%).

Compound 17: A solution of **5** (110 mg, 35 μmol) in dry CH_2Cl_2 (2 mL) was added dropwise to a solution of *N-tert*-butoxycarbonylbutylendiamine (**8**, 75 μL, 0.37 mmol, 1.5 equiv) in dry CH_2Cl_2 (1 mL). The mixture was stirred at RT for 16 h and the reaction mixture was quenched with water (10 mL) and extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were dried ($MgSO_4$), filtered, concentrated and purified by flash chromatography (20:1→15:1 CH_2Cl_2/MeOH). Yield: 114 mg (72%); R_f = 0.37 (9:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +65.8 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 6.89 (brs, 14H; NH), 5.25 (t, $J_{2,3}=J_{3,4}$ = 8.9 Hz, 7H; H-3), 5.08 (d, $J_{1,2}$ = 3.7 Hz, 7H; H-1), 4.95 (brs, 7H; NH), 4.75 (dd, 7H; H-2), 4.14 (m, 7H; H-5), 3.75 (t, $J_{4,5}$ = 8.8 Hz, 7H; H-4), 3.76 (m, 14H; CH_2N_{cyst}), 3.46 (m, 14H; CH_2NH), 3.19 (brd, $J_{6a,6b}$ = 12.2 Hz, 7H; H-6a), 3.10 (m, 14H; CH_2NHBoc), 3.03 (dd, $J_{5,6b}$ =

5.6 Hz, 7H; H-6b), 2.92, 2.80 (2dt, ^2J(H,H) = 13.3 Hz, ^3J(H,H) = 6.5 Hz, 14H; CH_2S_{cyst}), 2.38–2.30 (m, 14H; CH_2CO), 2.28–2.21 (m, 7H; CH_2CO), 2.18–2.11 (m, 7H; CH_2CO), 1.60–1.49 (m, 56H; CH_2CH$_2$CO, CH_2CH$_2$NH, CH_2CH$_2$NHBoc), 1.41 (s, 63H; CMe_3), 1.31–1.24 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.89, 0.87 ppm (2t, ^3J(H,H) = 7.4 Hz, ^3J(H,H) = 6.7 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 182.0 (CS), 173.4, 171.6 (CO ester), 156.4 (CO carbamate), 96.6 (C-1), 79.3 (CMe_3), 78.9 (C-4), 71.5 (C-5), 70.4 (C-2, C-3), 44.2 (CH_2NH), 43.8 (CH_2N_{cyst}), 40.3 (CH_2NHBoc), 34.1 (CH_2CO), 33.8 (C-6), 33.1 (CH_2S_{cyst}), 31.4, 31.2 ($CH_3CH_2CH_2$), 28.5 (CMe_3), 27.6 (CH_2CH$_2$NHBoc), 26.4 (CH_2CH$_2$NH), 24.4, 24.3 (CH_2CH$_2$CO), 22.3 (CH_3CH_2), 13.8 ppm (CH_3); ESIMS: *m*/*z*: 1533.9 $[M+3\,Na]^{3+}$, 2288.9 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{210}H_{371}N_{21}O_{56}S_{14}$: C 55.61, H 8.25, N 6.49; found: C 55.36, H 8.30, N 6.26.

Compound 18: A solution of **5** (110 mg, 35 μmol) in dry CH_2Cl_2 (2 mL) was added dropwise to a solution of *N-tert*-butoxycarbonylhexylendiamine (**9**, 82 μL, 0.37 mmol, 1.5 equiv) in dry CH_2Cl_2 (1 mL). The mixture was stirred at RT for 12 h and the reaction mixture was quenched with water (10 mL) and extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were dried ($MgSO_4$), filtered, concentrated, and purified by flash chromatography (20:1→15:1 CH_2Cl_2/MeOH). Yield: 107 mg (65%); R_f = 0.38 (9:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +65.6 (*c* = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 6.89 (brs, 7H; NH), 6.82 (brs, 14H; NH), 5.26 (t, $J_{2,3}$ = $J_{3,4}$ = 8.9 Hz, 7H; H-3), 5.09 (d, $J_{1,2}$ = 3.7 Hz, 7H; H-1), 4.76 (m, 7H; NH), 4.75 (dd, 7H; H-2), 4.15 (m, 7H; H-5), 3.76 (t, $J_{4,5}$ = 8.8 Hz, 7H; H-4), 3.72 (m, 14H; CH_2N_{cyst}), 3.43 (m, 14H; CH_2NH), 3.18 (brd, $J_{6a,6b}$ = 12.1 Hz, 7H; H-6a), 3.06 (m, 21H; CH_2NHBoc, H-6b), 2.92, 2.79 (2dt, ^2J(H,H) = 13.3 Hz, ^3J(H,H) = 6.7 Hz, 14H; CH_2S_{cyst}), 2.38–2.30 (m, 14H; CH_2CO), 2.28–2.21 (m, 7H; CH_2CO), 2.18–2.11 (m, 7H; CH_2CO), 1.56 (m, 42H; CH_2CH$_2$CO, CH_2CH$_2$NH), 1.45 (m, 14H; CH_2CH$_2$NHBoc), 1.41 (s, 63H; CMe_3), 1.31–1.24 (m, 84H; CH_3CH_2, $CH_3CH_2CH_2$, CH_2), 0.89, 0.87 ppm (2t, ^3J(H,H) = 7.3 Hz, ^3J(H,H) = 6.8 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 182.0 (CS), 173.5, 171.6 (CO ester), 156.2 (CO carbamate), 96.6 (C-1), 79.1 (CMe_3), 78.8 (C-4), 71.4 (C-5), 70.4 (C-2, C-3), 44.5 (CH_2NH), 43.8 (CH_2N_{cyst}), 40.5 (CH_2NHBoc), 34.1 (CH_2CO), 33.8 (C-6), 33.1 (CH_2S_{cyst}), 31.4, 31.3 ($CH_3CH_2CH_2$), 30.0 (CH_2CH$_2$NHBoc), 29.0 (CH_2CH$_2$NH), 28.5 (CMe_3), 26.5, 26.4 (CH_2), 24.4, 24.3 (CH_2CH$_2$CO), 22.3 (CH_3CH_2), 13.8 ppm (CH_3); ESIMS: *m*/*z*: 2388.4 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{224}H_{399}N_{21}O_{56}S_{14}$: C 56.86, H 8.50, N 6.22; found: C 56.61, H 8.3, N 6.10.

Compound 19: A solution of 4-(*tert*-butoxycarbonylaminomethyl)benzyl isothiocyanate[55] (**12**, 44.3 mg, 160 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL) was added to a solution of **HexCD-N** (66 mg, 20.7 μmol) and Et_3N (22 μL, 160 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 85 mg (84%); R_f = 0.48 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +66.9 (*c* = 1.1 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.10 (m, 28H; Ph), 6.95, 6.83 (2brs, 14H; NHCS), 5.25 (t, $J_{2,3}$ = $J_{3,4}$ = 9.1 Hz, 7H; H-3), 5.16 (brs, 7H; NHBoc), 5.09 (d, $J_{1,2}$ = 3.7 Hz, 7H; H-1), 4.74 (dd, 7H; H-2), 4.48 (brs, 14H; PhCH_2NHCS), 4.15 (m, 21H; H-5, CH_2NHBoc), 3.73 (t, $J_{4,5}$ = 8.7 Hz, 7H; H-4), 3.64 (brs, 14H; CH_2CH$_2$S$_{cyst}$), 3.20 (brd, $J_{6a,6b}$ = 13.2 Hz, 7H; H-6a), 3.01 (dd, $J_{6b,5}$ = 5.5 Hz, 7H; H-6b), 2.80 (2dt, ^2J(H,H) = 13.4 Hz, ^3J(H,H) = 6.6 Hz, 14H; CH_2S_{cyst}), 2.40–2.10 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.40 (s, 63H; CMe_3), 1.28 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.89, 0.88 ppm (2t, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): δ = 182.5 (CS), 173.9, 172.0 (CO ester), 156.7 (CO carbamate), 138.8, 128.3, 127.9, 127.5 (Ph), 97.0 (C-1), 80.0 (CMe_3), 79.4 (C-4), 72.0 (C-5), 70.8 (C-3, C-2), 48.4 (PhCH_2NCS), 44.8 (PhCH_2NHBoc), 44.2 (CH_2CH$_2$S$_{cyst}$), 34.5, 34.2 (CH_2CO, C-6), 33.4 (CH_2S_{cyst}), 31.8, 31.6 ($CH_3CH_2CH_2$), 28.8 (CMe_3), 24.8, 24.7 (CH_2CH$_2$CO), 22.7 (CH_3CH_2), 14.2 ppm (CH_3); ESIMS: *m*/*z*: 2454.8 $[M+K+H]^{2+}$, 1643.5 $[M+K+Na+H]^{3+}$; elemental analysis calcd (%) for $C_{238}H_{371}N_{21}O_{56}S_{14}$: C 58.68, H 7.68, N 6.04; found: C 58.76, H 7.60, N 5.88.

Compound 20: A solution of 3-(*tert*-butoxycarbonylaminomethyl)benzyl isothiocyanate[55] (**13**, 44.3 mg, 160 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL) was added to a solution of 5 (66 mg, 20.7 μmol) and Et_3N (22 μL, 160 μmol, 1.1 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 90 mg (88%); R_f = 0.48 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +63.2 (*c* = 1.1 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.18 (t, ^3J(H,H) = 9.4 Hz, 7H; H-5$_{Ph}$), 7.10 (d, ^3J(H,H) = 9.4 Hz, 7H; H-6$_{Ph}$), 7.05 (d, ^3J(H,H) = 9.4 Hz, 7H; H-4$_{Ph}$), 7.01 (s, 7H; H-2$_{Ph}$), 6.95 (brs, 14H; NHCS), 5.24 (t, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, 7H; H-3), 5.18 (brs, 7H; NHBoc), 5.09 (d, $J_{1,2}$ = 3.8 Hz, 7H; H-1), 4.75 (dd, 7H; H-2), 4.45 (brs, 14H; PhCH_2NHCS), 4.14 (m, 21H; H-5, PhCH_2NHBoc), 3.75 (t, $J_{4,5}$ = 9.0 Hz, 7H; H-4), 3.70 (brs, 14H; CH_2CH$_2$S$_{cyst}$), 3.21 (brd, $J_{6a,6b}$ = 13.1 Hz, 7H; H-6a), 3.04 (dd, $J_{5,6b}$ = 5.5 Hz, 7H; H-6b), 2.82 (m, 14H; CH_2S_{cyst}), 2.40–2.10 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.40 (s, 63H; CMe_3), 1.28 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.89–0.88 ppm (m, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 182.5 (CS), 173.7, 171.9 (CO ester), 156.7 (CO carbamate), 139.8, 138.3, 129.1, 126.8, 126.4, 126.0 (Ph), 96.9 (C-1), 80.0 (CMe_3), 79.2 (C-4), 71.8 (C-5), 70.7 (C-3, C-2), 48.4 (PhCH_2NCS), 44.8 (PhCH_2NHBoc), 44.2 (CH_2CH$_2$S$_{cyst}$), 34.3, 34.1 (CH_2CO, C-6), 33.2 (CH_2S_{cyst}), 31.7, 31.5 ($CH_3CH_2CH_2$), 28.7 (CMe_3), 24.7, 24.6 (CH_2CH$_2$CO), 22.6 (CH_3CH_2), 14.1 ppm (CH_3); ESIMS: *m*/*z*: 2455.3 $[M+K+H]^{2+}$, 1649.9 $[M+K+2Na]^{3+}$; elemental analysis calcd (%) for $C_{238}H_{371}N_{21}O_{56}S_{14}$: C 58.68, H 7.68, N 6.04; found: C 58.62, H 7.66, N 5.85.

Compound 21: A solution of **14**[56] (89 mg, 259 μmol, 1.2 equiv) in CH_2Cl_2 (2 mL) was added to a solution of **HexCD-N** (98 mg, 31 μmol) and Et_3N (33 μL, 238 μmol, 1.1 equiv) in CH_2Cl_2 (2 mL), . The reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). R_f = 0.48 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +62.8 (*c* = 0.8 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.42 –6.90 (m, 14H; NHCS), 5.27 (t, $J_{2,3}$ = $J_{3,4}$ = 8.9 Hz, 7H; H-3), 5.08 (d, $J_{1,2}$ = 3.7 Hz, 7H; H-1), 5.06 (brs, 7H; NHBoc), 4.75 (dd, 7H; H-2), 4.15 (m, 7H; H-5), 3.80 (t, $J_{4,5}$ = 8.9 Hz, 7H; H-4), 3.70 (brs, 14H; CH_2CH$_2$S$_{cyst}$), 3.62 (brs, 14H; BocNCH_2CH$_2$NHCS), 3.39 (m, 14H; CSNHCH_2CH$_2$NBoc), 3.32 (m, 14H; BocNHCH$_2$CH$_2$NBoc), 3.23 (brq, ^3J(H,H) = 5.4 Hz, 14H; CH_2NHBoc), 3.17 (brd, $J_{6a,6b}$ = 10.9 Hz, 7H; H-6a), 3.08 (brd, 7H; H-6b), 2.85 (m, 14H; CH_2S_{cyst}), 2.40–2.10 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.45 (s, 63H; NHCOOCMe_3), 1.41 (s, 63H; NCOOCMe_3), 1.32 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.91, 0.89 ppm (2t, ^3J(H,H) = 7.6 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 179.8 (CS), 173.6, 171.8 (CO ester), 156.4 (CO carbamate), 96.8 (C-1), 80.8 (CMe_3), 78.8 (C-4), 71.6 (C-5), 70.7 (C-3, C-2), 58.1 (BocNCH_2CH$_2$NHBoc), 57.5 (BocNCH_2CH$_2$NHCS), 44.0 (CH_2CH$_2$S$_{cyst}$), 43.5 (NCH_2CH$_2$NHCS), 40.1 (CH_2NHBoc), 34.3, 34.0 (CH_2CO, C-6), 33.0 (CH_2S_{cyst}), 31.6, 31.5 ($CH_3CH_2CH_2$), 28.7 (CMe_3), 24.6 (CH_2CH$_2$CO), 22.5 (CH_3CH_2), 14.0 ppm (CH_3); ESIMS: *m*/*z*: 2691.9 $[M+2Na]^{2+}$, 1808.3 $[M+2Na+K]^{3+}$; elemental analysis calcd (%) for $C_{245}H_{434}N_{28}O_{70}S_{14}$: C 55.09, H 8.19, N 7.34; found: C 54.79, H 8.09, N 7.23.

Compound 22: A solution of **15**[56] (61 mg, 158 μmol, 1.2 equiv) in CH_2Cl_2 (1.5 mL) was added to a solution of **HexCD-N** (60 mg, 18.8 μmol) and Et_3N (37 μL, 265 μmol, 2.0 equiv) in CH_2Cl_2 (1.5 mL), and the reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 90 mg (85%); R_f = 0.46 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +57.4 (*c* = 1.0 in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$, 313 K): δ = 7.22, 7.16 (2brs, 14H; NHCS), 5.29 (t, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, 7H; H-3), 5.24 (brs, 14H; NHBoc), 5.08 (d, $J_{1,2}$ = 3.5 Hz, 7H; H-1), 4.77 (dd, 7H; H-2), 4.17 (m, 7H; H-5), 3.83 (t, $J_{4,5}$ = 9.2 Hz, 7H; H-4), 3.77 (brq, 14H; CH_2CH$_2$S$_{cyst}$), 3.54 (brq, 14H; NCH_2CH$_2$NHCS), 3.15 (m, 42H; H-6a, H-6b, CH_2NHBoc), 2.87 (m, 14H; CH_2S_{cyst}), 2.67 (brt, ^3J(H,H) = 5.5 Hz, 14H; NCH_2CH$_2$NHCS), 2.58 (brt, ^3J(H,H) = 5.2 Hz, 28H; CH_2CH$_2$NHBoc), 2.43–2.11 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.44 (s, 126H; CMe_3), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.91, 0.89 ppm (2t, ^3J(H,H) = 7.8 Hz, 42H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): δ = 182.8 (CS), 173.8, 172.0 (CO ester), 156.9 (CO carbamate), 97.0 (C-1), 79.8 (CMe_3), 79.0 (C-4), 71.8 (C-5), 70.9 (C-3, C-2), 55.1 (CH_2CH$_2$NHBoc), 54.0 (NCH_2CH$_2$NHCS), 44.5 (CH_2CH$_2$S$_{cyst}$), 42.9 (NCH_2CH$_2$NHCS), 39.5 (CH_2NHBoc), 34.4, 34.2

(CH_2CO, C-6), 33.6 (CH_2S_{cyst}), 31.8, 31.7 ($CH_3CH_2CH_2$), 28.9 (CMe_3), 24.8, 24.7 (CH_2CH_2CO), 22.7 (CH_3CH_2), 14.2 ppm (CH_3); ESIMS: m/z: 2820.3 $[M+2H]^{2+}$, 1880.7 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{259}H_{469}N_{35}O_{70}S_{14}$: C 55.13, H 8.38, N 8.69; found: C 54.76, H 8.21, N 8.50.

Compound 23: A solution of **5** (50 mg, 15.5 μmol) in CH_2Cl_2 (2.5 mL) was added to a solution of bis(2-(*tert*-butoxycarbonylamino)ethyl)-amine[30e] (**10**, 36 mg, 120 μmol, 1.1 equiv) and Et_3N (17 μL, 122 μmol, 1.1 equiv) in CH_2Cl_2 (2.5 mL), and the reaction mixture was stirred at RT for 16 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 83 mg (99%); R_f = 0.41 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +57.0 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, MeOD, 313 K): δ = 5.30 (t, $J_{2,3}$ = $J_{3,4}$ = 8.6 Hz, 7H; H-3), 5.13 (d, $J_{1,2}$ = 3.4 Hz, 7H; H-1), 4.79 (dd, 7H; H-2), 4.17 (m, 7H; H-5), 3.93 (t, $J_{4,5}$ = 8.6 Hz, 7H; H-4), 3.86 (m, 14H; $CH_2CH_2S_{cyst}$), 3.74 (m, 28H; CH_2NCS), 3.20 (m, 42H; H-6a, H-6b, CH_2NHBoc), 2.98 (m, 14H; CH_2S_{cyst}), 2.40–2.10 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH_2CO), 1.44 (s, 126H; CMe_3), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.91, 0.89 ppm (2t, ^3J(H,H) = 7.8 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 313 K): δ = 181.6 (CS), 173.6, 172.5 (CO ester), 157.6 (CO carbamate), 97.2 (C-1), 80.3 (CMe_3), 79.0 (C-4), 72.1 (C-5), 71.0 (C-3, C-2), 50.9 (CH_2NCS), 45.9 ($CH_2CH_2S_{cyst}$), 38.4 (CH_2NHBoc), 34.2, 34.0 (CH_2CO, C-6), 32.8 (CH_2S_{cyst}), 31.7, 31.5 ($CH_3CH_2CH_2$), 28.9 (CMe_3), 24.7 (CH_2CH_2CO), 22.6 (CH_3CH_2), 13.5 ppm (CH_3); ESIMS: m/z: 2701.3 $[M+Na+K]^{2+}$, 1813.9 $[M+Na+2K]^{3+}$; elemental analysis calcd (%) for $C_{245}H_{434}N_{28}O_{70}S_{14}$: C 55.09, H 8.19, N 7.34; found: C 54.83, H 8.08, N 7.26.

Compound 24: Compound **11**[56] (68 mg, 261 μmol) was added to a solution of **5** (100 mg, 31 μmol) and Et_3N (36 μL, 261 μmol) in CH_2Cl_2 (3 mL), and the solution was stirred at RT for 16 h. The solvent was evaporated under diminished pressure and the residue was purified by flash chromatography (20:1 CH_2Cl_2/MeOH). Yield: 115 mg (73%); $[\alpha]_D$ = +63.4 (c = 1.0 in CH_2Cl_2); R_f = 0.74 (6:1 CH_2Cl_2/MeOH); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.52–7.13 (m, 28H; NHCS), 5.44 (brs, 14H; NHBoc), 5.30 (t, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, 7H; H-3), 5.12 (d, $J_{1,2}$ = 3.5 Hz, 7H; H-1), 4.82 (dd, 7H; H-2), 4.19 (m, 7H; H-5), 3.83 (t, $J_{4,5}$ = 9.2 Hz, 7H; H-4), 3.90–3.50 (m, 56H; CH_2NHCS), 3.30 (m, 21H; H-6a, CH_2NHBoc), 3.10 (m, 7H; H-6b), 2.93 (m, 14H; CH_2S_{cyst}), 2.50–2.13 (m, 28H; CH_2CO), 1.75–1.55 (m, 28H; CH_2CH_2CO), 1.44 (s, 63H; CMe_3), 1.40–1.20 (m, 56H; $CH_3CH_2CH_2$, CH_3CH_2), 1.00–0.97 ppm (m, 42H; CH_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): δ = 182.8 (CS), 173.8, 172.0 (CO ester), 156.9 (CO carbamate), 97.0 (C-1), 79.8 (CMe_3), 79.0 (C-4), 71.8 (C-5), 70.9 (C-2, C-3), 55.1 (NCH_2CH_2NHBoc), 54.0 (NCH_2CH_2NHCS), 44.5 (SCH_2CH_2NHCS), 42.9 (NCH_2CH_2NHCS), 39.5 (CH_2NHBoc), 34.3 (CH_2CO, C-6), 33.6 (SCH_2CH_2NHCS), 31.7 ($CH_3CH_2CH_2$), 28.9 (CMe_3), 24.7 (CH_2CH_2CO), 22.7 (CH_3CH_2), 14.2 ppm (CH_3). ESIMS: m/z: 2546.2 $[M+2Na]^{2+}$, 1705.9 $[M+3Na]^{3+}$; elemental analysis calcd (%) for $C_{217}H_{385}N_{35}O_{56}S_{21}$: C 51.57, H 7.68, N 9.70; found: C 51.39, H 7.46, N 9.46.

Compound 25: Acetic anhydride (3 mL) was added to a solution of **2** (0.56 g, 0.25 mmol) in pyridine (6 mL), and the reaction was stirred at RT for 24 h. Then, the mixture was poured into ice water (30 mL), the product was extracted with CH_2Cl_2 (2×20 mL), the organic layer was washed successively with 2N H_2SO_4 (2×20 mL) and saturated $NaHCO_3$ (2×20 mL), dried with Na_2SO_4, and the solvent was removed under diminished pressure. The resulting residue was purified by flash chromatography (40:1→20:1 CH_2Cl_2/MeOH). Yield: 0.58 g (81%); R_f = 0.80 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +18.9 (c = 1.0 in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 5.39 (brs, 7H; NH), 5.28 (t, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, 7H; H-3), 5.15 (d, $J_{1,2}$ = 3.9 Hz, 7H; H-1), 4.84 (dd, 7H; H-2), 4.20 (m, 7H; H-5), 3.82 (t, $J_{4,5}$ = 9.4 Hz, 7H; H-4), 3.63 (brq, ^3J(H,H) = 6.5 Hz, 14H; CH_2N), 3.16 (dd, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6a}$ = 2.1 Hz, 7H; H-6a), 3.06 (dd, $J_{5,6b}$ = 6.0 Hz, 7H; H-6b), 2.82 (m, 14H; CH_2S_{cyst}), 2.06, 2.02 (2s, 42H; MeCO), 1.42 ppm (s, 63H; CMe_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): δ = 171.1, 169.8 (CO ester), 156.4 (CO carbamate), 97.1 (C-1), 79.7 (C-4), 79.4 (CMe_3), 71.7 (C-3), 71.3 (C-2), 70.9 (C-5), 40.6 (CH_2N_{cyst}), 34.1 (CH_2S_{cyst}, C-6), 28.9 (CMe_3), 21.2 ppm ($MeCO$); ESIMS: m/z: 2860.5 $[M+Na]^+$, 1442.0 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{119}H_{189}N_7O_{56}S_7$: C 50.63, H 6.71, N 3.45; found: C 50.21, H 6.46, N 3.35.

Compound 26: Treatment of **25** (56 mg, 20 μmol) with a mixture of TFA/CH_2Cl_2 (1:1, 2 mL) at RT for 2 h, followed by evaporation of the solvents at diminished pressure afforded in virtually quantitative yield **26** (58 mg), which was characterized as its heptakis(trifluoroacetate). $[\alpha]_D$ = +51.7 (c = 1.0 in water); ^{1}H NMR (500 MHz, MeOD, 313 K): δ = 5.38 (t, $J_{2,3}$ = $J_{3,4}$ = 8.0 Hz, 7H; H-3), 5.24 (d, $J_{1,2}$ = 4.0 Hz, 7H; H-1), 4.82 (dd, 7H; H-2), 4.14 (m, 7H; H-5), 3.96 (t, 7H; H-4), 3.20 (m, 14H; H-6), 3.12 (t, ^3J-(H,H) = 7.0 Hz, 14H; CH_2N), 3.06 (t, 14H; CH_2S_{cyst}), 2.10, 2.08 ppm (2s, 42H; MeCO); ^{13}C NMR (125.7 MHz, MeOD, 313 K): δ = 172.0, 171.9 (CO), 97.9 (C-1), 79.9 (C-4), 73.8 (C-3), 72.2 (C-2), 71.9 (C-5), 40.6 (CH_2N_{cyst}), 34.8 (C-6), 31.8 (CH_2S_{cyst}), 21.2, 20.9 ppm ($MeCO$); ESIMS: m/z: 2136.4 $[M+H]^+$; 1069.2 $[M+2H]^{2+}$; elemental analysis calcd (%) for $C_{98}H_{140}F_{21}N_7O_{56}S_7{\cdot}2H_2O$: C 39.61, H 4.88, N 3.30, S 7.65; found: C 39.57, H 4.85, N 3.09, S 7.49.

Compound 28: A solution of 2-(*tert*-butoxycarbonylamino)ethylisothiocyanate (**27**, 34 mg, 0.17 mmol, 1.2 equiv) in CH_2Cl_2 (1.5 mL) was added to a solution of **26** (58 mg, 20 μmol) and Et_3N (29 μL, 0.21 mmol, 1.5 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred at RT for 24 h. The solvent was evaporated under diminished pressure. The residue was purified by flash chromatography (50:1→20:1 CH_2Cl_2/MeOH). Yield: 50 mg (70%); R_f = 0.37 (20:1 CH_2Cl_2/MeOH); $[\alpha]_D$ = +69.0 (c = 0.4 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$, 313 K): δ = 7.20, 7.01 (2brs, 14H; NH thiourea), 5.44 (brs, 7H; NH carbamate), 5.24 (t, $J_{2,3}$ = $J_{3,4}$ = 8.8 Hz, 7H; H-3), 5.10 (d, $J_{1,2}$ = 3.6 Hz, 7H; H-1), 4.78 (dd, 7H; H-2), 4.17 (m, 7H; H-5), 3.75 (t, $J_{4,5}$ = 8.5 Hz, 7H; H-4), 3.74, 3.58 (2brs, 28H; CH_2NHCS), 3.29 (brq, ^3J(H,H) = 5.6 Hz, 14H; CH_2NHBoc), 3.21 (brd, $J_{6a,6b}$ = 13.0 Hz, 7H; H-6a), 3.03 (dd, $J_{5,6b}$ = 7.6 Hz, 7H; H-6b), 2.94, 2.82 (2m, 14H; CH_2S_{cyst}), 2.05, 2.00 (2s, 42H; MeCO), 1.42 ppm (s, 63H; CMe_3); ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K): δ = 182.6 (CS), 171.0, 169.6 (CO ester), 157.2 (CO carbamate), 97.2 (C-1), 80.2 (C-4), 79.7 (CMe_3), 72.0 (C-3), 71.3 (C-2), 70.9 (C-5), 45.4, 45.0 (CH_2NHCS), 40.5 (CH_2NHBoc), 34.1 (CH_2S_{cyst}), 33.3 (C-6), 28.9 (CMe_3), 21.0 ppm ($MeCO$); ESIMS: m/z: 1818.8 $[M+2K]^{2+}$, 1806.8 $[M+Na+K]^{2+}$, 1798.8 $[M+2Na]^{2+}$; elemental analysis calcd (%) for $C_{140}H_{231}N_{21}O_{56}S_{14}$: C 47.32, H 6.55, N 8.28, S 12.63; found: C 47.41, H 6.62, N 8.15, S 12.48.

Compound 29: A solution of **28** (50 mg, 14 μmol) in MeOH (3 mL) was treated with methanolic NaOMe (1M, 25 μL). The reaction mixture was stirred at RT for 16 h, then neutralized with Amberlite IR-120 (H^+) ion-exchange resin, the solution was filtered, and the solvent was removed under diminished pressure. Yield: 42 mg (99%); R_f = 0.59 (10:2:1 MeCN/water/NH_4OH); $[\alpha]_D$ = +34.9 (c = 0.24 in MeOH); ^{1}H NMR (500 MHz, MeOD, 323 K): δ = 4.99 (d, $J_{1,2}$ = 3.4 Hz, 7H; H-1), 4.05 (m, 7H; H-5), 3.81 (t, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, 7H; H-3), 3.73, 3.58 (2brs, 28H; CH_2NHCS), 3.50 (m, 14H; H-2, H-4), 3.26 (m, 21H; H-6a, CH_2NHBoc), 2.98 (m, 7H; H-6b), 2.92 (m, 14H; CH_2S_{cyst}), 1.46 ppm (brs, 63H; CMe_3); ^{13}C NMR (125.7 MHz, MeOD, 323 K): δ = 183.7 (CS), 158.6 (CO), 104.1 (C-1), 86.4 (C-4), 80.6 (C_q), 74.5 (C-3), 74.4 (C-2), 73.5 (C-5), 45.3, 45.2 (CH_2NHCS), 41.2 (CH_2NHBoc), 35.0 (CH_2S_{cyst}), 33.8 (C-6), 29.1 ppm (CMe_3); ESIMS: m/z: 1501.8 $[M+H+K]^{2+}$; elemental analysis calcd (%) for $C_{112}H_{203}N_{21}O_{42}S_{14}$: C 45.37, H 6.90, N 9.92; found: C 45.11, H 6.69, N 9.80.

Compound CD-T-C_2N: Compound **29** (42 mg, 17 μmol) was treated with a mixture of TFA/CH_2Cl_2 (1:1, 2 mL) at RT for 2 h. Then the solvents were evaporated under diminished pressure and further coevaporated with water. The resulting residue was freeze dried from diluted HCl to afford **CD-T-C_2N** (32 mg) in virtually quantitative yield as its heptahydrochloride. $[\alpha]_D$ = +46.5 (c = 0.5 in H_2O); ^{1}H NMR (500 MHz, D_2O, 333 K): δ = 5.42 (d, $J_{1,2}$ = 3.1 Hz, 7H; H-1), 4.25 (brt, $J_{4,5}$ = 9.1 Hz, 7H; H-5), 4.20 (t, $J_{2,3}$ = $J_{3,4}$ = 9.1 Hz, 7H; H-3), 4.14 (t, ^3J(H,H) = 6.0 Hz, 14H; $CH_2CH_2NH_2$), 3.99 (brs, 14H; CH_2NHCS), 3.96 (dd, 7H; H-2), 3.87 (t, 7H; H-4), 3.55 (t, ^3J(H,H) = 6.0 Hz, 14H; CH_2NH_2), 3.52 (brd, $J_{6a,6b}$ = 13.8 Hz, 7H; H-6a), 3.28 (dd, $J_{5,6b}$ = 7.8 Hz, 7H; H-6b), 3.21 ppm (t, ^3J-(H,H) = 6.9 Hz, 14H; CH_2S_{cyst}); ^{13}C NMR (125.7 MHz, D_2O, 333 K): δ = 182.8 (CS), 101.6 (C-1), 84.1 (C-4), 73.6 (C-3), 72.5 (C-2), 72.0 (C-5), 44.3, 41.6 (CH_2NHCS), 39.8 (CH_2NH_2), 34.1 (C-6), 32.4 ppm (CH_2S_{cyst}); ESIMS: m/z: 1132.8 $[M+2H]^{2+}$; elemental analysis calcd (%) for

$C_{77}H_{154}Cl_7N_{21}O_{28}S_{14}$: C 36.71, H 6.16, N 11.68, S 17.82; found: C 36.48, H 5.95, N 11.44, S 17.49.

Compound HexCD-T-C_2N: Compound **16** (104 mg, 24 μmol) was dissolved in a mixture of TFA/CH_2Cl_2 (1:1, 2 mL) and stirred for 2 h. The acid was eliminated by repeated coevaporation with water and the resulting residue was dissolved in diluted HCl and freeze-dried to give pure **HexCD-T-C_2N**. Yield: 90 mg (96%); $[\alpha]_D = +80.7$ ($c = 1.0$ in DMSO); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 333 K): $\delta = 8.08$ (brs, 2H; NH_2), 7.92 (brs, 1H; NH), 7.72 (brs, 1H; NH), 5.21 ((t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 7H; H-3), 5.05 (d, $J_{1,2} = 2.9$ Hz, 7H; H-1), 4.67 (dd, 7H; H-2), 4.12 (m, 7H; H-5), 3.85 (t, $J_{4,5} = 8.6$ Hz, 7H; H-4), 3.68 (m, 14H; CH_2NH), 3.59 (m, 14H; CH_2N_{Cyst}), 3.10 (d, 14H; H-6a, H-6b), 2.98 (t, ^3J(H,H) = 6.3 Hz, 14H; CH_2NH_2), 2.79, 2.77 (2dt, ^2J(H,H) = 13.5 Hz, ^3J(H,H) = 7.0 Hz, 14H; CH_2S_{Cyst}), 2.35–2.11 (m, 28H; CH_2CO), 1.50 (m, 28H; CH_2CH$_2$CO), 1.24 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.84, 0.83 ppm (2t, ^3J(H,H) = 7.3 Hz, ^3J(H,H) = 6.9 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, [D$_6$]DMSO, 333 K): $\delta = 183.4$ (CS), 172.9, 171.9 (CO), 96.7 (C-1), 78.5 (C-4), 71.5 (C-5), 70.7 (C-3, C-2), 44.2 (CH_2N_{Cyst}), 41.7 (CH_2NH), 39.0 (CH_2NH_2), 34.0 (C-6), 33.7 (CH_2CO), 32.9 (CH_2S_{Cyst}), 31.3, 31.1 ($CH_3CH_2CH_2$), 24.3, 24.2 (CH_2CH$_2$CO), 22.2 (CH_3CH_2), 14.0 ppm (CH_3); ESIMS: m/z: 1819.4 $[M+2H]^{2+}$, 1213.3 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{161}H_{294}Cl_7N_{21}O_{42}S_{14}$: C 49.67, H 7.61 N 7.56; found: C 49.27 H 7.30 N 7.32.

Compound HexCD-T-C_4N: Treatment of **17** (58 mg, 13 μmol) with TFA/CH_2Cl_2 (1:1, 1 mL) at RT for 2 h, followed by evaporation of the solvents and freeze-drying from a diluted HCl solution, gave pure **HexCD-T-C_4N**. Yield: 52.8 mg (99%); $[\alpha]_D = +46.9$ ($c = 0.5$ in DMSO); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 333 K): $\delta = 7.89$ (brs, 14H; NH_2), 7.68 (brs, 7H; NH), 7.54 (brs, 7H; NH), 5.21 (t, $J_{3,4} = 9.0$ Hz, 7H; H-3), 5.04 (d, $J_{1,2} = 3.1$ Hz, 7H; H-1), 4.67 (dd, $J_{2,3} = 9.9$, Hz, 7H; H-2), 4.11 (m, 7H; H-5), 3.85 (t, $J_{4,5} = 8.5$ Hz, 7H; H-4), 3.58 (m, 14H; CH_2N_{cyst}), 3.37 (m, 14H; CH_2NH), 3.10 (m, 14H; H-6a, H-6b), 2.76 (m, 28H; CH_2NH_2, CH_2S), 2.34–2.29 (m, 14H; CH_2CO), 2.21–2.11 (m, 14H; CH_2CO), 1.58–1.48 (m, 56H; CH_2CH$_2$CO, CH_2CH$_2$NH, CH_2CH$_2$NH$_2$), 1.25–1.22 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.84, 0.83 ppm (2t, ^3J(H,H) = 7.0 Hz, ^3J(H,H) = 6.8 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, [D$_6$]DMSO, 313 K): $\delta = 183.1$ (CS), 172.9, 171.9 (CO), 96.7 (C-1), 78.5 (C-4), 71.6 (C-5), 70.7 (C-2, C-3), 44.1 (CH_2NH_{yst}), 43.5 (CH_2NH), 39.2 (CH_2NH_2), 33.9, 33.7 (C-6, CH_2CO), 33.1 (CH_2S_{cyst}), 31.3, 31.1 ($CH_3CH_2CH_2$), 26.3, 25.0 (CH_2CH$_2$NH, CH_2CH$_2$NH$_2$), 24.3, 24.2 (CH_2CH$_2$CO), 22.2 (CH_3CH_2), 14.0 ppm (CH_3); ESIMS: m/z: 1917.3 $[M+2H]^{2+}$, 1278.5 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{175}H_{322}Cl_7N_{21}O_{42}S_{14}$: C 51.40, H 7.94, N 7.19; found: C 51.31, H 7.75, N 6.96.

Compound HexCD-T-C_6N: Treatment of **18** (33.4 mg, 7 μmol) with TFA/CH_2Cl_2 (1:1, 1 mL) at RT for 2 h, followed by evaporation of the solvents and freeze-drying from a diluted HCl solution, gave pure **HexCD-T-C_6N**. Yield: 30 mg (99%); $[\alpha]_D = +56.9$ ($c = 0.3$ in DMSO); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 333 K): $\delta = 7.79$ (brs, 14H; NH_2), 7.54 (brs, 7H; NH), 7.43 (brs, 7H; NH), 5.22 (t, $J_{3,4} = 9.0$ Hz, 7H; H-3), 5.04 (d, $J_{1,2} = 3.0$ Hz, 7H; H-1), 4.67 (dd, $J_{2,3} = 10.0$ Hz, 7H; H-2), 4.11 (m, 7H; H-5), 3.85 (t, $J_{4,5} = 8.5$ Hz, 7H; H-4), 3.58 (m, 14H; CH_2N_{cyst}), 3.34 (m, 14H; CH_2NH), 3.08 (m, 7H; H-6a, H-6b), 2.75 (m, 28H; CH_2NH_2, CH_2S_{cyst}), 2.34–2.29 (m, 14H; CH_2CO), 2.21–2.11 (m, 14H; CH_2CO), 1.55–1.45 (m, 56H; CH_2CH$_2$CO, CH_2CH$_2$NH, CH_2CH$_2$NH$_2$), 1.29–1.22 (m, 84H; CH_3CH_2, $CH_3CH_2CH_2$, CH_2), 0.84, 0.83 ppm (2t, ^3J(H,H) = 6.9 Hz, ^3J(H,H) = 6.5 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, [D$_6$]DMSO, 333 K): $\delta = 182.9$ (CS), 172.9, 171.9 (CO), 96.7 (C-1), 78.4 (C-4), 71.6 (C-5), 70.6 (C-2, C-3), 44.1 (CH_2NH, CH_2N_{cyst}), 39.4 (CH_2NH_2), 33.9, 3.7 (C-6, CH_2CO), 33.2 (CH_2S_{cyst}), 31.3, 31.1 ($CH_3CH_2CH_2$), 29.1 (CH_2CH$_2$NH), 27.4 (CH_2CH$_2$NH$_2$), 26.4, 26.1 (CH_2), 24.3, 24.2 (CH_2CH$_2$CO), 22.2 (CH_3CH_2), 14.0 ppm (CH_3); ESIMS: m/z: 2015.3 $[M+2H]^{2+}$, 1343.8 $[M+3H]^{3+}$; elemental analysis calcd (%) for $C_{189}H_{350}Cl_7N_{21}O_{42}S_{14}$: C 52.96, H 8.23, N 6.86; found: C 53.02, H 8.18, N 6.63.

Compound HexCD-T-*p*XN: Treatment of **19** (30 mg, 6.2 μmol) with TFA/CH_2Cl_2 (1:1, 0.6 mL) at RT for 2 h, followed by evaporation of the solvents and freeze-drying from a diluted HCl solution, gave pure **HexCD-T-*p*XN**. Yield: 27 mg (99%); $[\alpha]_D = +54.9$ ($c = 1.0$ in MeOH); ^{1}H NMR (500 MHz, MeOD, 313 K): $\delta = 7.35$ (2d, ^3J(H,H) = 7.2 Hz, 28H; Ar), 5.32 (t, $J_{2,3} = J_{3,4} = 8.8$ Hz, 7H; H-3), 5.15 (d, $J_{1,2} = 3.6$ Hz, 7H; H-1), 4.80 (dd, 7H; H-2), 4.67 (brs, 14H; PhCH_2NHCS), 4.17 (m, 21H; H-5), 4.06 (s, 14H; PhCH_2NH_2), 3.90 (t, $J_{4,5} = 8.8$ Hz, 7H; H-4), 3.72 (brs, 14H; C$H_2CH_2S_{cyst}$), 3.27 (brd, $J_{6a,6b} = 14.2$ Hz, 7H; H-6a), 3.16 (dd, $J_{5,6b} = 5.2$ Hz, 7H; H-6b), 2.90 (m, 14H; CH_2S_{cyst}), 2.50–2.20 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH_2CO), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.90, 0.89 ppm (2t, ^3J(H,H) = 7.2 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 313 K): $\delta = 184.0$ (CS), 174.8, 173.5 (CO), 141.3, 130.1, 129.3, 129.0 (Ph), 98.2 (C-1), 80.0 (C-4), 73.2 (C-5), 71.9 (C-3), 71.7 (C-2), 49.4 (PhCH_2NCS), 45.3 (C$H_2CH_2S_{cyst}$), 44.1 (PhCH_2NH_2), 35.2, 35.1 (CH_2CO, C-6), 34.1 (CH_2S_{cyst}), 32.6, 32.4 ($CH_3CH_2CH_2$), 25.6 (CH_2CH$_2$CO), 23.5 (CH_3CH_2), 14.2 ppm (CH_3); ESIMS: m/z: 2085.2 $[M+2H]^{2+}$, 1390.4 $[M+3H]^{3+}$, 1043.0 $[M+4H]^{4+}$; elemental analysis calcd (%) for $C_{203}H_{329}N_{21}O_{42}S_{14}Cl_{14}$: C 55.09, H 7.33, N 6.65; found: C 54.89, H 7.396, N 6.508.

Compound HexCD-T-m*p*XN: Treatment of **20** (30 mg, 6.2 μmol) with TFA/CH_2Cl_2 (1:1, 0.6 mL) at RT for 2 h, followed by evaporation of the solvents and freeze-drying from a diluted HCl solution, gave pure **HexCD-T-*m*XN**. Yield: 27 mg (99%); $[\alpha]_D = +47.8$ ($c = 1.0$ in MeOH); ^{1}H NMR (500 MHz, MeOD, 323 K): $\delta = 7.40$ (m, 28H; Ar), 5.36 (t, $J_{2,3} = J_{3,4} = 8.7$ Hz, 7H; H-3), 5.19 (d, $J_{1,2} = 3.0$ Hz, 7H; H-1), 4.85 (dd, 7H; H-2), 4.77 (brs, 14H; PhCH_2NHCS), 4.18 (m, 21H; H-5, PhCH_2NH_2), 3.95 (t, $J_{4,5} = 8.7$ Hz, 7H; H-4), 3.79 (brs, 14H; C$H_2CH_2S_{cyst}$), 3.31 (brd, 7H; H-6a), 3.20 (brd, $J_{6b,6b} = 13.0$ Hz, 7H; H-6b), 2.94 (m, 14H; CH_2S_{cyst}), 2.50–2.20 (m, 28H; CH_2CO), 1.66 (m, 28H; CH_2CH$_2$CO), 1.35 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.96–0.94 ppm (m, ^3J(H,H) = 7.0 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 323 K): $\delta = 182.4$ (CS), 173.4, 172.1 (CO), 140.0, 133.1, 129.1, 128.0, 127.7, 127.3 (Ph), 96.9 (C-1), 78.7 (C-4), 71.9 (C-5), 70.6 (C-3), 70.3 (C-2), 48.4 (PhCH_2NCS), 44.1 (C$H_2CH_2S_{cyst}$), 43.1 (PhCH_2NH_2), 33.8, 33.7 (CH_2CO, C-6), 32.8 (CH_2S_{cyst}), 31.2, 31.1 ($CH_3CH_2CH_2$), 24.2 (CH_2CH$_2$CO), 24.2 (CH_3CH_2), 12.9 ppm (CH_3); ESIMS: m/z: 2085.2 $[M+2H]^{2+}$, 1390.4 $[M+3H]^{3+}$, 1043.0 $[M+4H]^{4+}$, 883.6 $[M+5H]^{5+}$; elemental analysis calcd (%) for $C_{203}H_{329}N_{21}O_{42}S_{14}Cl_{14}$: C 55.09, H 7.33, N 6.65; found: C 54.88, H 7.29, N 6.24.

Compound HexCD-T-C_2N-C_2N: Treatment of the carbamate **21** (45.5 mg, 8.55 μmol) with 1:2 TFA/CH_2Cl_2 at RT for 2 h, followed by evaporation of the solvent and freeze-drying from diluted HCl solution, gave pure **HexCD-T-C_2N-C_2N**. Yield: 38 mg (99%); $R_f = 0.05$ (6:3:1 MeCN/H_2O/NH_4OH); $[\alpha]_D = +29.4$ ($c = 1.0$ in MeOH); ^{1}H NMR (500 MHz, 5:1 MeOD/D_2O, 313 K): $\delta = 5.30$ (t, $J_{2,3} = J_{3,4} = 8.7$ Hz, 7H; H-3), 5.14 (d, $J_{1,2} = 3.54$ Hz, 7H; H-1), 4.80 (dd, 7H; H-2), 4.15 (m, 7H; H-5), 3.97 (brs, 14H; HNCH$_2$CH_2NHCS), 3.89 (t, $J_{4,5} = 8.7$ Hz, 7H; H-4), 3.75 (brs, 14H; C$H_2CH_2S_{cyst}$), 3.50 (t, ^3J(H,H) = 6.3 Hz, 14H; CH_2NH_2), 3.46 (t, 14H; NH$_2$CH$_2$CH_2NH), 3.41 (t, ^3J(H,H) = 6.0 Hz, 14H; CSNHCH$_2$CH_2NH), 3.25 (brd, 7H; H-6a), 3.14 (m, 7H; H-6b), 2.91 (m, 14H; CH_2S_{cyst}), 2.50–2.15 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.30 (m, 56H; $CH_3CH_2CH_2$, CH_3CH_2), 0.91, 0.88 ppm (2t, ^3J(H,H) = 6.7 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 323 K): $\delta = 184.3$ (CS), 173.6, 172.2 (CO), 96.8 (C-1), 78.8 (C-4), 71.9 (C-5), 70.6 (C-3), 70.3 (C-2), 57.5 (HNCH_2CH$_2$NHCS), 44.7 (CH_2NH_2), 44.1 (C$H_2CH_2S_{cyst}$), 40.2 (NHCH$_2$CH_2NHCS), 35.7 (NHCH_2CH$_2$NHBoc), 33.9 (C-6), 33.8 (CH_2CO), 32.6 (CH_2S_{cyst}), 31.2, 31.0 ($CH_3CH_2CH_2$), 24.2 (CH_2CH$_2$CO), 22.1, 22.0 (CH_3CH_2), 13.1, 12.9 ppm (CH_3); ESIMS: m/z: 1968.9 $[M+2H]^{2+}$, 1313.4 $[M+3H]^{3+}$, 985.6 $[M+4H]^{4+}$, 788.7 $[M+5H]^{5+}$; elemental analysis calcd (%) for $C_{175}H_{336}N_{28}O_{42}S_{14}Cl_{14}$: C 47.23, H 7.61, N 8.81; found: C 47.13, H 7.66, N 8.29.

Compound HexCD-T-C_2N[C_2N]$_2$: Treatment of the carbamate **22** (30 mg, 5.3 μmol) with 1:1 TFA/CH_2Cl_2 at RT for 2 h, followed by evaporation of the solvent and freeze-drying from diluted HCl solution, gave pure **HexCD-T-C_2N[C_2N]$_2$**. Yield: 25 mg (99%); $[\alpha]_D = +48.2$ ($c = 0.67$ in MeOH); ^{1}H NMR (500 MHz, 5:1 MeOD/D_2O, 333 K): $\delta = 5.27$ (t, $J_{2,3} = J_{3,4} = 8.6$ Hz, 7H; H-3), 5.13 (d, $J_{1,2} = 3.7$ Hz, 7H; H-1), 4.82 (dd, 7H; H-2), 4.14 (m, 7H; H-5), 3.89 (t, $J_{4,5} = 8.6$ Hz, 7H; H-4), 3.73 (m, 28H; C$H_2CH_2S_{cyst}$, NCH$_2$CH_2NHCS), 3.25 (t, ^3J(H,H) = 6.2 Hz, 28H; CH_2NH_2), 3.14 (m, 14H; H-6a, H-6b), 3.14 (t, 28H; CH_2CH$_2$NH$_2$), 2.97 (t, ^3J(H,H) = 6.5 Hz, 28H; NCH_2CH$_2$NHCS), 2.90 (m, 14H; CH_2S_{cyst}), 2.50–2.15 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH$_2$CO), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.91, 0.89 ppm (2t, ^3J(H,H) = 7.8 Hz, 42H; CH_3);

^{13}C NMR (125.7 MHz, 5:1 MeOD-D_2O, 333 K): δ = 183.5 (CS), 175.0, 173.4 (CO), 98.1 (C-1), 80.1 (C-4), 73.1 (C-5), 71.9 (C-3), 71.5 (C-2), 53.5 (NCH_2CH_2NHCS), 52.3 ($CH_2CH_2NH_2$), 45.4 ($CH_2CH_2S_{cyst}$), 42.1 (NCH_2CH_2NHCS), 38.1 (CH_2NH_2), 35.2 (C-6), 35.0 (CH_2CO), 33.9 (CH_2S_{cyst}), 32.3, 32.2 ($CH_3CH_2CH_2$), 25.4, 25.3 (CH_2CH_2CO), 23.2, 23.1 (CH_3CH_2), 14.2 ppm (CH_3); ESIMS: *m/z*: 2119.6 $[M+2H]^{2+}$, 1413.8 $[M+3H]^{3+}$, 1060.3 $[M+4H]^{4+}$, 848.7 $[M+5H]^{5+}$, 707.3 $[M+6H]^{6+}$, 606.3 $[M+7H]^{7+}$; elemental analysis calcd (%) for $C_{189}H_{371}N_{35}O_{42}S_{14}Cl_{14}$: C 47.78, H 7.87, N 10.32; found: C 47.45, H 7.34, N 10.35.

Compound HexCD-T[C_2N]$_2$: Treatment of the carbamate **23** (30 mg, 5.6 μmol) with 1:1 TFA/CH_2Cl_2 at RT for 2 h, followed by evaporation of the solvent and freeze-drying from diluted HCl solution, gave pure **HexCD-T[C_2N]$_2$**. Yield: 25 mg (99%); $[\alpha]_D = +19.5$ (c = 0.85 in MeOH); ^{1}H NMR (500 MHz, MeOD, 313 K): δ = 5.32 (t, $J_{2,3} = J_{3,4} = 8.9$ Hz, 7H; H-3), 5.13 (d, $J_{1,2} = 3.6$ Hz, 7H; H-1), 4.77 (dd, 7H; H-2), 4.14 (m, 7H; H-5), 4.02 (m, 56H; CH_2NCS), 3.91 (t, $J_{4,5} = 8.6$ Hz, 7H; H-4), 3.84 (t, ^3J(H,H) = 6.9 Hz, 14H; $CH_2CH_2S_{cyst}$), 3.25 (t, ^3J(H,H) = 6.9 Hz, 56H; CH_2NH_2), 3.22 (br d, $J_{6a,6b} = 13.2$ Hz, 7H; H-6a), 3.22 (dd, $J_{5,6b} = 5.3$ Hz, 7H; H-6b), 2.94 (t, 14H; CH_2S_{cyst}), 2.50–2.15 (m, 28H; CH_2CO), 1.60 (m, 28H; CH_2CH_2CO), 1.30 (m, 56H; CH_3CH_2, $CH_3CH_2CH_2$), 0.93, 0.92 ppm (2t, ^3J(H,H) = 8.2 Hz, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 313 K): δ = 182.8 (CS), 173.4, 172.1 (CO), 96.8 (C-1), 78.5 (C-4), 71.9 (C-5), 70.5 (C-3), 70.4 (C-2), 47.1 (CH_2NCS), 46.1 ($CH_2CH_2S_{cyst}$), 36.9 (CH_2NH_2), 33.9 (C-6), 33.8, 33.7 (CH_2CO), 32.2 (CH_2S_{cyst}), 31.2, 31.1 ($CH_3CH_2CH_2$), 24.2 (CH_2CH_2CO), 22.1 (CH_3CH_2), 13.2, 13.1 ppm (CH_3); ESIMS: *m/z*: 1969.4 $[M+2H]^{2+}$; elemental analysis calcd (%) for $C_{175}H_{336}N_{28}O_{42}S_{14}Cl_{14}$: C 47.23, H 7.61, N 8.81; found: C 47.64, H 7.59, N 8.85.

Compound HexCD-T-C_2-T-C_2N: Treatment of the carbamate **24** (30 mg, 5.9 μmol) with 1:1 TFA/CH_2Cl_2 (2 mL) at RT for 2 h, followed by evaporation of the solvent and freeze-drying from diluted HCl solution, gave pure **HexCD-T-C_2-T-C_2N**. Yield: 21 mg (99%); $[\alpha]_D = +53.7$ (c = 1.0 in MeOH); ^{1}H NMR (500 MHz, MeOD, 313 K): δ = 5.35 (br t, $J_{2,3} = J_{3,4}$ = 8.9 Hz, 7H; H-3), 5.19 (d, $J_{1,2} = 3.0$ Hz, 7H; H-1), 4.85 (dd, 7H; H-2), 4.21 (m, 7H; H-5), 3.94 (m, 21H; H-4, $CH_2CH_2NH_2$), 3.79 (br s, 14H; $CH_2CH_2S_{Cyst}$), 3.73 (br s, 28H; $CSNHCH_2CH_2NHCS$, $CSNHCH_2CH_2NHCS$), 3.28 (m, 28H; CH_2NH_2, H-6a), 2.98 (m, 14H; CH_2S_{Cyst}), 2.50–2.20 (m, 28H; CH_2CO), 1.75–1.55 (m, 28H; CH_2CH_2CO), 1.50–1.30 (m, 56H; $CH_3CH_2CH_2$, CH_3CH_2), 1.01–0.90 ppm (m, 42H; CH_3); ^{13}C NMR (125.7 MHz, MeOD, 313 K): δ = 186.3, 184.7 (CS), 176.0, 174.7 (CO), 99.4 (C-1), 81.3 (C-4), 74.5 (C-5), 73.2 (C-3), 72.9 (C-2), 46.6 ($SCNHCH_2CH_2NH_2$), 45.8 ($SCNHCH_2CH_2NHCS$, $SCNHCH_2CH_2NHCS$), 43.9 (CH_2NH_2), 42.3 ($CH_2CH_2S_{Cyst}$), 36.4 (CH_2CO, C-6), 35.5 (CH_2S_{Cyst}), 33.7 ($CH_3CH_2CH_2$), 26.9 (CH_2CH_2CO), 24.8 (CH_3CH_2), 15.6 ppm (CH_3); ESIMS: *m/z*: 2176.2 $[M+2H]^{2+}$, 1451.0 $[M+3H]^{3+}$, 1088.5 $[M+4H]^{4+}$; elemental analysis calcd (%) for $C_{182}H_{338}N_{35}O_{42}S_{21}Cl_7$: C 47.43, H 7.35, N 5.39; found: C 47.11, H 7.23, N 10.39.

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