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Hydrogen-Bonded Self-Assembled Peptide Nanotubes from Cystine-Based Macrocyclic Bisureas

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Abstract: The design, synthesis, and characterization of amino acid-based cyclic bisureas, a new class of macrocyclic peptides, are described. These cystine-based macrocycles are constructed by a single-step procedure involving condensation of 1,ω-alkane diisocyanate[$(CH_2)_n(NCO)_2$; $n = 4, 6, 12$] with either the simple L-cystine dimethyl ester to provide simple cyclic bisureas of 16-, 18-, and 24-membered rings through $1 + 1$ cyclization or with extended cystine bispeptides leading to a variety of cystine-based macrocyclic peptide bisureas. The potential of cyclic bisureas to serve as artificial receptors for 1,ω-alkane dicarboxylic acids has been demonstrated with 18- and 24-membered macrocycles which show specific binding with dianions of oxalic and succinic acid, respectively. Single-crystal X-ray studies have shown these cyclic bisureas to possess an inherent property of self-assembling into vertical stacks of tube-like structures. The hollow, open-ended tubes offer enormous scope as models for studying biological phenomena or for designing materials with novel electronic and optical properties.

Creation of hollow tubular structures by noncovalent self-assembly of appropriately crafted organic molecules has been the subject of considerable research in recent years.[1] Organic nanotubes constructed from amino acid-based macrocycles are particularly important because of their potential utility as models for mimicking biological channels or as transport vehicles in drug delivery systems.[2]

Rational design of macrocycles that can be persuaded to form tube-like structures by stacking atop one another through hydrogen bonds is a challenge that is still only partially met. Ghadiri *et al.*[3] have utilized amide bonds of peptides to create extended cyclic structures. The channel structure is formed from

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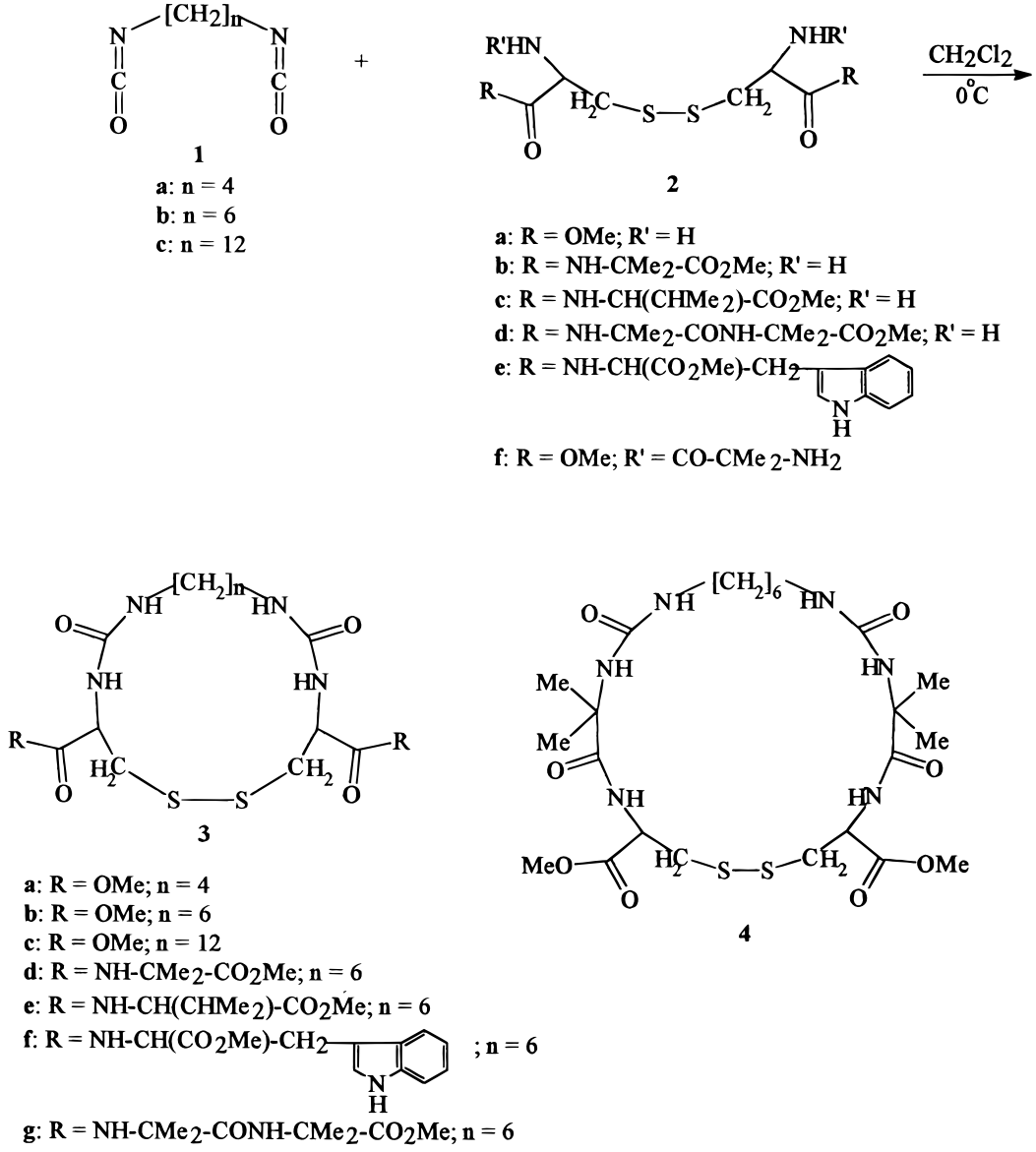

Figure 1. Synthesis of cystine-based cyclic bisureas.

a relatively simple individual unit, a macrocyclic peptide, constructed from an even number of alternating D- and L-amino acids, adopting a flat-ring conformation in which all backbone amide functionalities lie approximately perpendicular to the plane of the ring with side chain oriented outward. The peptide rings in this conformation are poised to form contiguously hydrogen-bonded β-sheetlike tubular ensembles. The design however does not permit cyclic peptides of less than 24-membered rings to form vertical stacks. In an alternate approach, cyclic peptides, constructed from either a combination of equal number of α- and β-amino acids[4] or all β-amino acids,[5] have been demonstrated to assemble into tube-like structures through contiguous amide−amide hydrogen bonding between macrocycles. The flexibility of β-amino acids allows the vertical stacking of rings as small as 14-membered. Our recent work[6] has shown that flat-ring conformation can also be achieved by incorporating small aromatic units into the cyclic backbone. Thus, 18-membered macrocycles containing an alternating sequence of L-serine and aromatic (Ph or Pyr) units were demonstrated to adopt relatively flat-ring conformation and formed tubular structures using aromatic $\pi-\pi$ interaction as the main organizing force.

Although urea functionality has been utilized for creating highly organized, hydrogen-bonded assemblies of chains,[7] ribbons,[8] and layers,[9] no report of a tubular ensemble, created by urea-type hydrogen bonding is known. We envisaged that urea functional groups, if placed at appropriate positions in a macrocycle, would participate in contiguous urea-type complementary hydrogen bonding that may lead to extended channel-like structures. Such urea-bonded channels are likely to be more

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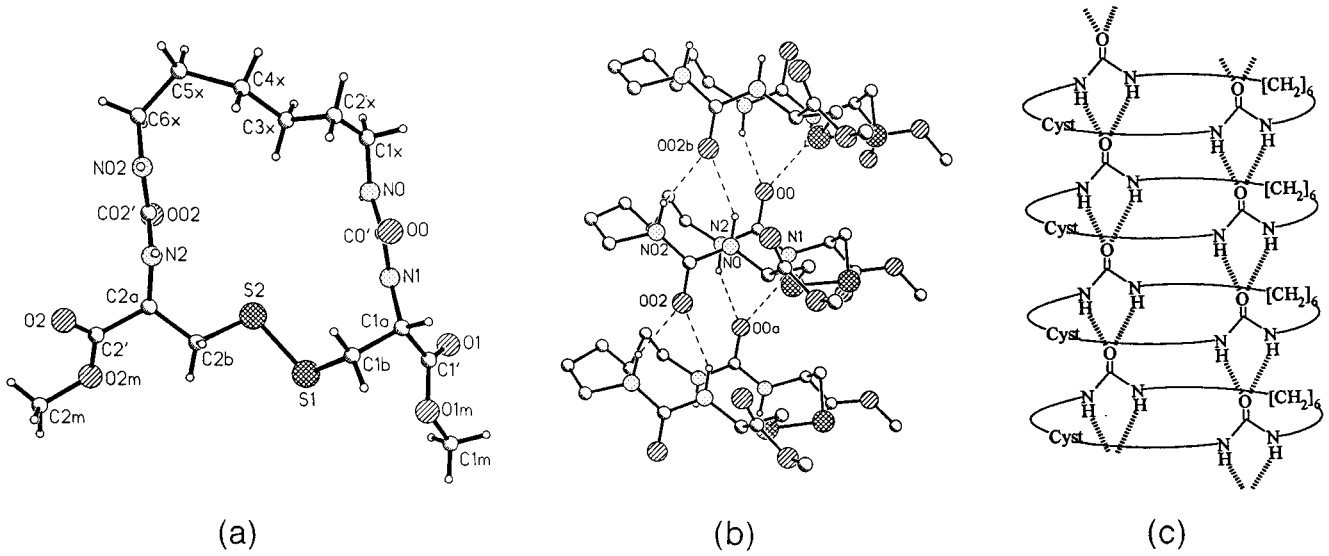


Figure 2. (a) X-ray structure of **3b**; Crystallographic data: $C_{16}H_{28}N_4O_6S_2$, space group $P1$, $a = 4.695(3)$ Å, $b = 10.488(2)$ Å, $c = 12.485(2)$ Å, $\alpha = 66.96(1)°$, $\beta = 85.73(2)°$, $\gamma = 78.84(2)°$, $V = 550.04$ g/cm^3, $d_{calc} = 1.306$ g/cm^3, $R_1 = 10.3$ for 2113 data observed with $F_o > 4\sigma$, Cu Kα radiation. The structure was solved by direct phase determination. The aliphatic chain segment from C1x to C6x is very flexible and assumes a number of conformations in the crystal in a disordered manner. Two principal conformations (shown in Figure 4) were used in the F^2 least-squares refinement. Only one conformation is shown in Figures 2 and 3. Coordinates, bond lengths, and bond angles are deposited in the Cambridge Crystallographic Data File. Both ester groups extend outward. Cavity size: 5.82 [(N(02)−N(0)] × 5.16 Å (S2−C3x); some important torsional angles: C(1b)−S1−S2−C(2b) −100°, S2−S1−C(1b)−C(1a) −94°, S1−S2−C(2b)−C(2a) −171°, N1−C(1a)−C(1b)−S1 +69°, N2−C(2a)−C(2b)−S2 −70°, ϕ_1 −157°, ψ_1 −164°, ϕ_2 −111°, ψ_2 +170°; (b) In the vertical stack of **3b**, the molecules are aligned in a parallel fashion and participate in contiguous backbone−backbone urea-type hydrogen bonding, making a string of hydrogen bonds on either side of the stack, leading to the generation of an extended, open-ended tubular structure with hollow interior. All hydrogen bonds have N···O distances of 2.93−2.98 Å and H···O distances of 2.11−2.17 Å. The inter-ring distance (the distance at which the subunit repeats itself in the nanotube) is 4.69 Å. (c) Schematic picture of the hydrogen-bonded stack; Cyst = $CH(CO_2CH_3)CH_2-S-S-CH_2-CH(CO_2CH_3)$.

stable and may exhibit novel optical behavior. We provide here the first illustration of this concept and report on the design, synthesis, and crystal structure of cystine-based macrocyclic bisureas, a new class of cyclic peptides containing appropriately spaced urea functions in the backbone.

The single-step synthetic strategy (Figure 1) for macrocyclic bisureas involves the condensation of 1,ω-alkane diisocyanate [$(CH_2)_n(NCO)_2$; **1a**−**c**] with either the simple L-cystine dimethyl ester (**2a**) or its extended C,C′- or *N*,*N*′-bispeptides (**2b**−**e** and **2f**, respectively) under high dilution conditions to provide a large variety of cystine-based macrocyclic bisureas through 1 + 1 cyclizations. The ring size of the cyclic bisurea can be adjusted by choosing diisocyanate of appropriate length as illustrated here with the preparation of macrocycles (**3a**−**c**) ranging in ring size from 16- to 24-membered. The design is flexible with respect to the nature of the amino acid and permits the incorporation of a variety of amino acid residues either as part of the ring as in the case of macrocycle **4** or attached as pendants on the exterior of the ring as shown by the preparation of Aib-, Val-, and Trp-containing macrocycles **3d**, **3e**, and **3f** respectively. The pendant can even be a peptide unit as in the case of **3g** containing a pair of dipeptide (Aib-Aib-OMe) units on the outer rim of the macrocycle.

The diisocyanate, irrespective of whether $n = 4$, 6, or 12 (**1a**−**c**), yielded 1 + 1 bisurea (**3a**−**g**, **4**) as the only cyclic product. The cyclization product was, however, invariably accompanied by polymeric products arising from the linear oligomerization of the two components. The product bisureas were purified on a short column of silica gel by using a gradient elution with either hexane/ethyl acetate or chloroform/methanol solvents. The electrospray mass spectrometry confirmed their cyclic monomeric (1 + 1) nature. In ^{1}H NMR ($CDCl_3$), the urea amide protons in macrocycles **3a**−**c** consistently appeared as a pair of distinct resonances at ∼5.3 (multiplet) and ∼5.8 (doublet) ppm attributed to alkane NH and cystine NH, respectively. The same trend was maintained in amino acid-containing bisureas (**3a**−**f** and **4**). There was no indication of any intramolecular hydrogen bonding in any of the macrocyclic bisureas as examined by ^{1}H NMR solvent titration studies. The presence of an intense positive band at 208 nm in the CD spectrum (in trifluoroethanol at 298 K) suggested β-turn features. The cyclic structure breaks down in the presence of dithiothreitol as shown by the near absence of the 208 nm band.

Suitable crystals for X-ray diffraction were obtained for 18-membered cyclic bisurea **3b** from a mixture of chloroform and methanol. The crystal structure of **3b** (Figure 2a) showed that the 18-membered macrocycle, with all of the ester groups projecting outward, does not possess any internal hydrogen bonds. While all urea carbonyls in **3b** are oriented outward, the NH groups face inward. The crown-shaped ring of **3b** adopts an open-ring conformation with a relatively large cavity (5.82 × 5.16 Å) which is hollow. The lack of any electron density in X-ray difference maps attests to the emptiness in the cavity. The crystal structure further revealed that the bisurea rings aligned in a parallel fashion stack atop one another, maintaining proper registry between the subunits, generating an open-ended tube which extends to infinity. The hollow tubular-ensemble is held on either side by a string of hydrogen bonds which are of typical urea-type (Figure 2b). Figure 2c presents the schematic picture of the assembly. The four-sided hollow tube has three rigid walls, formed by the urea-type hydrogen bonding in two walls and the stacked S−S moiety in the third. The fourth wall, from C1x to C6x is very flexible, containing a number of conformations for the $(CH_2)_n$ segment. Only one conformation is shown in Figures 2 and 3. Two major conformations, for which the atomic sites were found among the strongest peaks in the X-ray difference map, shown in Figure 4, were used in

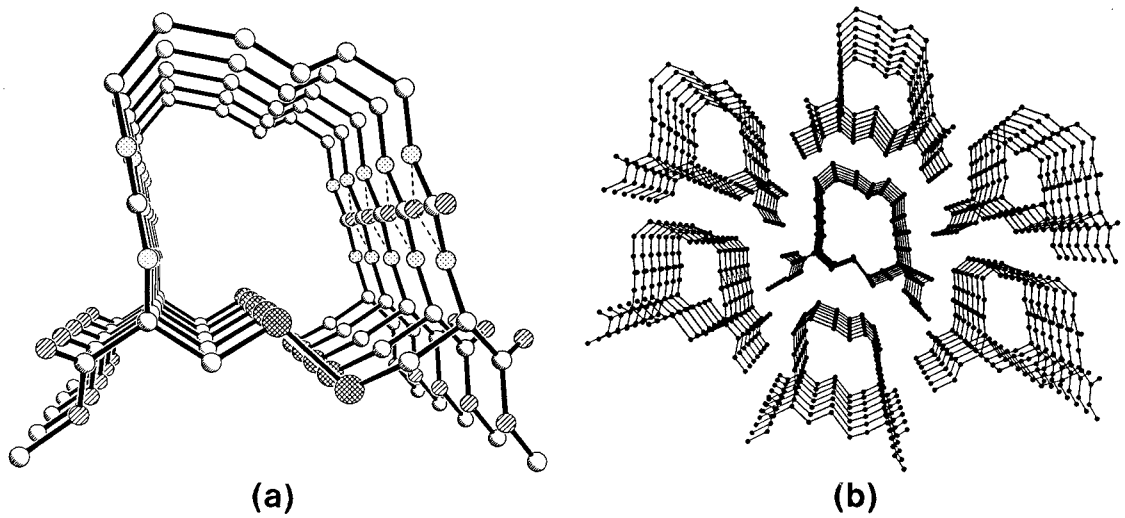


Figure 3. (a) View into the cavity of **3b** tube. The stack of 18-membered rings is held together by four urea-type hydrogen bonds between pairs of molecules. The cavity is empty. There is perfect registry between the molecules and stacking extends to infinity; (b) 3D view showing side-by-side stacking of bisurea tubes. The tubes are held in the pack only by hydrophobic interactions.

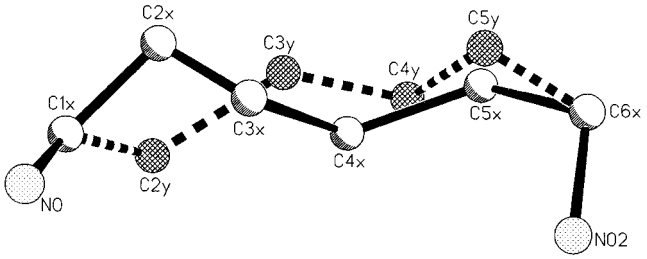
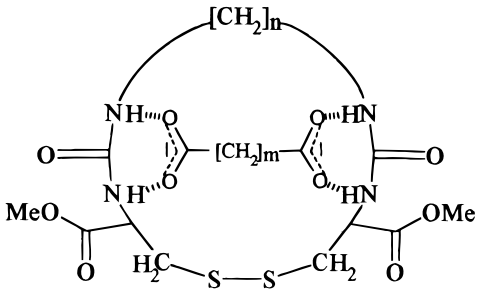


Figure 4. Two principal disorders in the aliphatic chain from C1x to C6x.

the least-squares refinement. However, a number of weak difference peaks, found in the same region between C1x and C6x, most likely account for additional conformations assumed by the hydrocarbon chain segment.

Figure 3a shows a view into the hydrogen-bonded cavity of **3b**. The neighboring tubes are held together only by hydrophobic interactions, Figure 3b.

The orientation of both pairs of urea NH groups into the interior of the cavity suggested that the 18-membered cyclic bisurea **3b** may serve as a tailor-made cyclic receptor for dicarboxylic acids.[10] The binding of **3b** with a variety of tertrabutylammonium (TBA) salts of 1,ω-alkane dicarboxylic acids [$(CH_2)_n(COOH)_2$; $n = 0, 1, ..., 4$] was examined by ^{1}H NMR studies. Interestingly, the host **3b**, only sparingly soluble in $CDCl_3$, was found to rapidly go into solution only upon the addition of oxalate guest, suggesting specificity for oxalate dianion. The macrocyclic bisurea host **3c** behaved similarly with only succinic acid TBA salt. There was considerable downfield shift of ~1.5 to 2 ppm of urea NH resonances in both **3b** and **3c** upon the addition of oxalate and succinate TBA salts, respectively.



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a: **n** = 12; **m** = 2
b: **n** = 6; **m** = 0

Figure 5. Bis-bidentate or tetrahydrogen-bonded complex of cyclic bisureas with 1,ω-alkane dicarboxylate dianions. The size complementarity leads to specific recognition of **3b** for oxalate and **3c** for succinate, respectively.

With the use of the NMR titration method,[11] the association constant (K_a) for **3b** with oxalate TBA salt and **3c** with succinate TBA salt in $CDCl_3$ at 298 K was measured as 6.21×10^3 M^{-1} and 2.7×10^3 M^{-1}, respectively. The proposed bis−bidentate or tetrahydrogen bonded structure (Figure 5) for **5a** and **5b** is supported by the maximum NH shift at a mole ratio of 1:1 (Supporting Information). The above molecular recognition studies have clearly shown that cyclic bisureas are excellent receptors for 1,ω-alkane dicarboxylic acids and would show specificity according to the size complementarity of the host−guest molecules.

In summary, we have introduced a new class of macrocyclic peptides, the cyclic bisureas, wherein the urea moieties are positioned at the two poles of the ring. The cyclic bisureas show an inherent tendency to self-assemble into tube-like structures by packing atop one another through contiguous urea-type hydrogen bonding on either side. The tubes are hollow, open-ended, with uniform shape and internal diameter and serve as

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(11) The association constant (K_a) was obtained by using the following equation: $K_{assoc} = \alpha/[(1 - \alpha)^2$ [c], where $\alpha = (\delta - \delta_0)/(\delta_{max} - \delta_0)$, δ_0 is the initial chemical shift (host alone), δ is the chemical shift at each titration point, and δ_{max} is the chemical shift when the receptor is entirely bound. [c] is the concentration of the guest or host. (Kelly, T. R.; Kim, M. H. *J. Am. Chem. Soc.* **1994**, *116*, 7072−7080 and references therein).

specific receptors for 1,ω-alkane dicarboxylic acids. The design permits a rigorous control over the pore diameter simply by adjusting the ring size of the bisurea subunit. The urea-based peptide tubes described here offer new structural and functional possibilities that may augment the potential utility of self-assembling peptide nanotubes in chemical, biological, and material science applications.

Experimental Section

All amino acids used were of L-configuration. 1,ω-Alkane diisocyanates were directly purchased from Aldrich Corp. Melting points were recorded on a Fisher-Jones melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker WM-300 instrument. Reactions were monitored wherever possible by thin layer chromatography. Silica gel (100−200 mesh, Merck) was used for column chromatography, and products were eluted with either a mixture of ethyl acetate/hexane or chloroform/methanol. The tetrabutylasmmonium salts of 1,ω-alkane dicarboxylic acids were prepared by adding 2 equiv of a 0.1 N solution of tetrabutylammonium hydroxide in methanol/toluene (SRL) in one portion to a stirred solution of the dicarboxylic acid (1 mmol) in dry methanol (∼2 mL). The resulting mixture after 2 h of stirring at room temperature was subjected to vacuum, and the thick syrupy residue was dried for 24 h over P_2O_5 in a vacuum desiccator. The dried bis-TBA salts were directly used for ^{1}H NMR experiments.

General Procedure for the Synthesis of Cyclic Bisureas. (a) Generation of Free Base of Simple Cystine diOMe. Cystine-diOMe dihydrochloride (3.41 g, 10 mmol) was admixed with an ice-cold saturated solution of sodium carbonate (∼20 mL) and stirred at 0 °C for ∼10 min. The pH of the reaction mixture was maintained at ∼9. The clear solution was extracted with dichloromethane (20 mL × 3), and the extract was dried (anhydrous $MgSO_4$), and evaporated in vacuo without heating. The residue (2.14 g, 80%) was used directly for the next reaction.

(b) Generation of Free Base of Cystine Bispeptides. An ice-cooled solution of the N$^\alpha$Boc-protected cystine bispeptide [(N$^\alpha$Boc)$_2$Cyst(Aaa-OMe)$_2$ or (N$^\alpha$BocAaa)$_2$−Cyst-diOMe or (N$^\alpha$Boc)$_2$Cyst(Aaa-Baa)$_2$; Aaa, Baa are the amino acid residues] in dry CH_2Cl_2 (2.5 mmol in 5 mL) was admixed with trifluoroacetic acid (6 mL) in dry CH_2Cl_2 (9 mL) (overall 30% TFA solution), and the reaction mixture that was left stirred at 0 °C for ∼2 h until no more starting material was left (TLC). The TFA solution was cooled in ice and directly treated first with saturated sodium carbonate solution (∼5 mL) followed by solid sodium carbonate until completely neutralized. The strongly basic solution was extracted with CH_2Cl_2 (20 mL × 3), dried (anhydrous $MgSO_4$), and evaporated in vacuo without heating. The residue (∼70% yield) was directly used for next reaction.

(c) Condensation of Free Base of Cystine-diOMe or Its Bispeptide with 1,ω-Alkane Diisocyanate. To a well-stirred and ice-cooled solution of the freshly generated cystine-based free base (a or b; 2 mmol in ∼500 mL dry CH_2Cl_2) was added dichloromethane solution of 1,ω-alkane diisocyanate [$(CH_2)_n(NCO)_2$; n = 4, 6, 12 etc] (2 mmol in ∼50 mL) dropwise over a period of ∼30−40 min. The reaction mixture that was left stirred at room temperature for 12 h. The solvents were evaporated using rotavapor, and the residue was chromatographed over a column of silica gel using a mixture of chloroform/methanol. The cyclic bisureas thus obtained were fully characterized.

Selective data: 3a: yield 16%; mp 221−223 °C; IR(KBr) 3346, 2962, 1748, 1634, 1587, 1445, 1357, 1317 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 1.21 (m, 4H), 1.57 (m, 2H), 2.63 (m, 2H), 2.87 (m, 2H), 3.07 (m, 2H), 3.65 (s, 6H), 4.52 (m, 2H), 6.13 (m, 2H), 6.38 (d, J = 8.4 Hz, 2H); ES-MS m/z (%) 431 (100) [M + Na$^+$], 409 (65) [M + H]$^+$.

3b: yield 35%; mp 219−221 °C; IR(KBr) 3346, 2928, 2854, 1748, 1640, 1580, 1431, 1344, 1317 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 1.23 (m, 4H), 1.49 (m, 4H), 2.82 (m, 2H), 3.20 (m, 2H), 3.41 (m, 4H), 3.75 (s, 6H), 4.89 (m, 2H), 5.97 (m, 2H), 6.43 (d, J = 7.5 Hz, 2H); ES-MS m/z (%) 437 (95) [M + H]$^+$.

3c: yield 20%; mp 206−208 °C; IR(KBr) 3339, 2935, 2854, 1742, 1640, 1573, 1445, 1344 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (m, 16H), 1.41 (m, 4H), 2.93 (m, 2H), 3.10 (m, 4H), 3.22 (m, 2H), 3.71 (s, 6H), 4.63 (m, 2H), 6.08 (m, 2H), 6.41 (d, J = 7.8 Hz, 2H); ES-MS m/z (%) 521 (100) [M + H]$^+$.

3d: yield 27%; mp 209−212 °C; IR(KBr) 3342, 3074, 2989 (sh), 2944, 2862, 1747, 1639, 1575, 1557, 1535, 1499, 1475, 1455, 1391, 1370 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (m, 20H), 2.87 (m, 2H), 3.15 (m, 4H), 3.38 (m, 2H), 3.67 (s, 6H), 4.81 (m, 2H), 5.88 (m, 2H), 6.40 (d, J = 8.4 Hz, 2H), 7.68 (s, 2H); ES-MS m/z (%) 607 (100) [M + H]$^+$.

3e: yield 20%; mp 199−203 °C; IR(KBr) 3330, 3089, 2941, 2860, 1744, 1711(sh), 1664(sh), 1641, 1575, 1547, 1525, 1469, 1443, 1377, 1311 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 0.93 (m, 12H), 1.31 (m, 2H), 1.50 (m, 6H), 2.16 (m, 2H), 2.87 (m, 2H), 3.18 (m, 2H), 3.34 (m, 4H), 3.70 (s, 6H), 4.42 (m, 2H), 4.76 (m, 2H), 6.14 (m, 2H), 6.58 (d, J = 7.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H); ES-MS m/z (%) 635 (100) [M + H]$^+$.

3f: yield 15%; mp 163−166 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (m, 8H), 2.91 (m, 4H), 3.20 (m, 4H), 3.27 (m, 4H), 3.65 (brs, 6H), 4.76 (m, 2H), 5.33 (m, 2H), 6.02 (br, 2H), 6.40 (br, 2H), 7.06 (m, 6H), 7.32 (m, 4H), 7.69 (br, 2H), 9.95 (br, 2H); FAB-MS m/z 809 (M + H)$^+$, 831 (M + Na$^+$)

3g: yield 55%; mp 125−130 °C; IR(KBr) 3387, 3059, 2994, 2940, 1742, 1667, 1645, 1575, 1557, 1539, 1520, 1447, 1390, 1368 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32−1.51 (m, 24H), 2.01 (m, 8H), 2.95 (m, 2H), 3.26 (m, 6H), 3.70 (s, 6H), 4.77 (m, 2H), 6.01 (brs, 2H), 6.61 (d, J = 7.5 Hz, 2H), 6.84 (s, 2H), 7.54 (s, 2H); ES-MS m/z (%) 777 (100) [M + H]$^+$.

4: yield 23%; mp 193−198 °C; IR(KBr) 3418 (sh), 3361, 3294 (sh), 2987 (sh), 2949, 2861, 1739, 1650, 1575, 1552, 1521, 1440, 1387, 1356, 1312 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 1.13−1.40 (m, 20H), 3.01−3.29 (m, 8H), 3.69 (s, 6H), 4.59 (m, 2H), 5.86 (m, 2H), 6.00 (s, 2H), 8.01 (d, J = 7.2 Hz, 2H); ES-MS m/z (%) 607 (100) [M + H]$^+$.

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Supporting Information Available: ^{1}H NMR spectra of **3b** (host) alone, of **3b** (host) with oxalate dianion (guest) in 1:1 molar ratio, of **3c** (host) alone, of **3c** (host) with succinate dianion (guest) in 1:1 molar ratio; graphs of chemical shift δ (NH1) with change in molar concentration of guest or host, of chemical shift δ (NH2) with change in molar concentration of guest or host; and crystal structure parameters for **3b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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