Parallel assembly of dipolar columns composed of a stacked cyclic tri-β-peptide

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Received 11th January 2006, Accepted 16th March 2006
First published as an Advance Article on the web 13th April 2006
DOI: 10.1039/b600407e

A novel cyclic trimer of a β-amino acid, *trans*-2-aminocyclohexylcarboxylic acid, was synthesized and its conformation and ability to form assemblies investigated. FT-IR and NMR measurements and computational calculations showed that this cyclic tri-β-peptide has a C_3-symmetric conformation with *trans* amide groups. A notable feature of the conformation is a vertical and parallel orientation of the three amide groups to the cyclic skeleton. The cyclic tri-β-peptide was crystallized from a solution in trifluoroacetic acid–methanol (or trifluoroacetic acid–water) to yield a rod-shaped molecular assembly, as observed by TEM. The electron crystallography of the rod-shaped assembly both in suspension and in ultrathin cross-section revealed that the cyclic tri-β-peptides were stacked up to form molecular columns, and that a two-fold screw symmetry operation along the column direction was present in the unit cell, which contained two cyclic tri-β-peptides. This indicates that all the amide groups are oriented in the same direction. Since any two molecular columns are staggered by a quarter of a *c*-axis length and aligned parallel to each other, the dipole moments of the columns are aligned to enhance the strength additively in the whole assembly.

Introduction

Peptides composed of β-amino acids have attracted much attention due to specific conformations that are frequently difficult to attain with peptides of α-amino acids. In particular, Seebach *et al.* and Gellman *et al.* have shown that homo-oligomers of chiral β-amino acids including *trans*-2-aminocyclohexylcarboxylic acid (*trans*-ACHC) had stable helical structures in solution.[1–8] β-Peptides are now actively applied to the fields of synthetic chemistry,[9–13] structural chemistry,[14–20] and biochemistry[21–25] because of these specific conformational properties. Recently, Seebach's group reported that cyclic tri-β-peptides such as cyclo(β-Hxx)$_3$ (xx = Ala, Glu, Glu(OBn), Ser(OBn), Met, where OBn represents a benzyl ester or ether) and cyclo(β-Asp(OBn))$_3$ formed a tubular structure in the solid state, as determined by an FT-IR study.[21,26–28] Further, cyclic tetra-β-peptides were synthesized by Seebach's and Ghadiri's groups, which also assembled into tubular structures through face-to-face molecular stacking *via* hydrogen bonds.[29,30] A unique feature of the molecular assemblies of cyclic trimers and tetramers is that amide groups in the cyclic skeleton are oriented perpendicularly to the ring plane, which promotes formation of intermolecular hydrogen bonds, to yield the tubular structure. Since all amide groups of cyclic tri-β-peptides orient in one direction within the tubular structure, the molecular assembly of cyclic trimers should possess a macrodipole moment. Since it has been pointed out that molecular dipole moments can influence the electron transfer reactions through a molecule,[31–37] we report in this paper the synthesis of a novel cyclic tri-β-peptide, with the aim of seeking out its possible applications to molecular devices.

In the smallest cyclic di-β-peptides, two amide groups are forced to take *cis*-form, which is not suitable for the present purpose.[38,39] We have therefore designed a novel cyclic trimer, cyclo(ACHC)$_3$, for the preparation of a tubular assembly. *Trans*-ACHC was chosen as the component, because the cyclohexane ring of ACHC is expected to promote the planar structure of the cyclic peptide, which should favor molecular stacking and the formation of a tubular structure. That is, when the C^{α}–C^{β} bond between the amino group and the carbonyl group in a β-amino acid is a part of ring skeleton, two bonds of C^{α}-amino group and C^{β}-carbonyl groups can take an equatorial orientation, leading to the stabilization of the flat conformation of the cyclic peptide. Furthermore, the cyclohexane ring may solve the solubility problem of cyclic β-peptides, which is often pointed out as a handling difficulty of β-peptides. It is also expected that the cyclic tri-β-peptide should be easily generated, because Gellman *et al.* demonstrated that polypeptides of ACHC took a 14-helix structure, in which about three residues of the β-amino acid make one turn, with amide groups orienting along the helical axis.[5] It is thus expected that cyclo(ACHC)$_3$ should spontaneously form a columnar structure through molecular stacking.

Results and discussion

Peptide synthesis and sample preparation

An optically active monomer, *trans*-(1*R*,2*R*)-(−)-ACHC, was synthesized from *trans*-(1*R*,2*R*)-(−)-cyclohexanedicarboxylic acid (*trans*-CHDC) according to the method reported in the literature.[40] Synthesis of the linear tripeptides, Boc-(ACHC)$_3$-OBn, was carried out according to a previous report.[5] After deprotection of a benzyl ester group from the C-terminus by Pd/C-catalyzed

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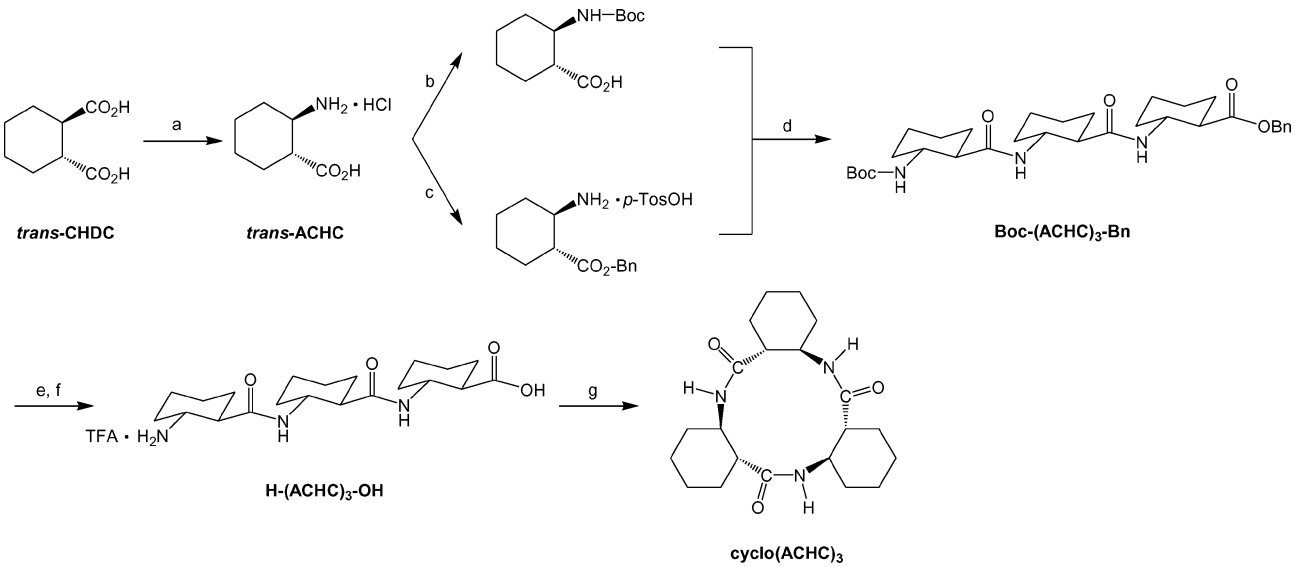

Scheme 1 Synthetic route for cyclo(ACHC)$_3$. *Reagents and conditions*: (a) see ref. 49; (b) Boc_2O, 1 N NaOH, dioxane–H_2O (1 : 1); (c) benzyl alcohol, *p*-TosOH, toluene, reflux; (d) stepwise elongation using HATU as a coupling reagent and TFA to deprotect the Boc group; (e) H_2, $Pd(OH)_2$, CH_2Cl_2; (f) TFA, anisole, CH_2Cl_2; (g) BOP, HOAT, DIEA.

hydrogenolysis and a Boc group from the N-terminal with trifluoroacetic acid (TFA) treatment, the cyclization reaction was carried out using 1-benzotriazoloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), 1-hydroxy-7-azabenzotriazole (HOAT) and diisopropylethylamine (DIEA) in dilute solution (Scheme 1). The obtained cyclic peptide was soluble only in TFA, in contrast to our expectation regarding improvement of solubility. It was expected that the molecular planarity of cyclo(ACHC)$_3$ should be enhanced by the presence of the cyclohexyl ring, resulting in good assembling properties (due to molecular stacking) to yield crystals easily. Cyclo(ACHC)$_3$ was purified successfully by recrystallization from TFA–methanol.

Samples of cyclo(ACHC)$_3$ with two crystal sizes were prepared for the purposes of analytical measurements. Large crystals of the rod-shaped assemblies with a diameter of a few μm were required for optical microscopy observations and electron diffraction (ED) analysis with an incident electron beam parallel to the column axis. In these cases, a crystal of cyclo(ACHC)$_3$ was dissolved in a small amount of TFA (1 mg ml^{-1}) and recrystallized slowly by a vapor diffusion method using methanol as the poor solvent. After standing for a few days, white needle-shaped crystals precipitated. In contrast, a finely crystalline sample was used for the transmission electron microscopy observations and ED analysis with an incident electron beam perpendicular to the column axis. A crystal of cyclo(ACHC)$_3$ was dissolved in a small amount of TFA (1 mg ml^{-1}), and ultra-pure water was added to the solution until it became slightly opaque, to obtain the molecular assembly as a suspension.

Conformation

In measurements of crystalline cyclo(ACHC)$_3$ by mass spectroscopy (FAB), signals corresponding to dimeric and trimeric compounds as well as the monomer appeared, suggesting its propensity to assemble spontaneously (*m*/*z* 751.51, calcd. for [M·M + H$^+$] *m*/*z* 751.61; *m*/*z* 1126.77, calcd. for [M·M·M + H$^+$] *m*/*z* 1126.76). The ^{13}C NMR spectrum of cyclo(ACHC)$_3$ in CF_3CO_2D–$CDCl_3$ showed carbon signals only for one unit of ACHC (δ/ppm: 177.3 (C=O), 55.2 (C1), 51.8 (C2), 31.9 (C3), 28.5 (C6), 24.4 (C4), 23.9 (C5)), indicating that cyclo(ACHC)$_3$ has a C_3-symmetric conformation on the NMR time scale.

An FT-IR spectrum of cyclo(ACHC)$_3$ in the solid state showed an amide I absorption (mainly C=O stretching mode) and an amide II absorption (mainly N–H bending and C–N stretching modes) at 1650 cm^{-1} and 1558 cm^{-1}, respectively. These wavenumbers are assigned to absorptions of a β-sheet-like tubular structure.[26–28,41–45] In particular, the amide I absorption at 1650 cm^{-1} suggests not anti-parallel β-sheets but parallel β-sheet structures.[1,28] Furthermore, a sharp N–H stretching (3288 cm^{-1}) peak suggests homogeneous hydrogen-bond formation in the cyclo(ACHC)$_3$ crystal. Taken together, the spectroscopic data indicate that crystalline cyclo(ACHC)$_3$ forms a "nanotube", similar to those in previous reports for cyclic tri-β-peptides of cyclo(β-Hxx)$_3$ (xx = Ala, Glu, Glu(OBn), Ser(OBn), Met)[21] and cyclo(β-Asp(OBn))$_3$.[26–28]

The conformation of cyclo(ACHC)$_3$ was studied further by geometry optimization with using geometry **1** (Fig. 1) as a starting conformation. Two optimized conformations (**A** and **B**) having a C_3 symmetry axis were obtained, as shown in Fig. 1, according to the method used for the optimization. The internal energy of conformation **A** (with *cis*-form amide groups, obtained by the AM 1 method[46]) is clearly higher than that of conformation **B** (with *trans*-form amide groups, optimized by an *ab initio* calculation using the Gaussian 03 program[47]); $\Delta U = 0.99$ eV. Furthermore, *cis*-amides of conformation **A** are incompatible with the results of the FT-IR measurements. Therefore, conformation **B**, in which cyclo(ACHC)$_3$ has a flat conformation with three *trans*-amide groups oriented perpendicular to the ring plane, is the most plausible conformation.

Seebach *et al.* reported that cyclo(β-HAla)$_3$ in a saturated solution of LiCl in tetrahydrofuran-d_8 showed a spin coupling constant between the amide proton and H$^\beta$ of 10.0 Hz.[27] Unfortunately,

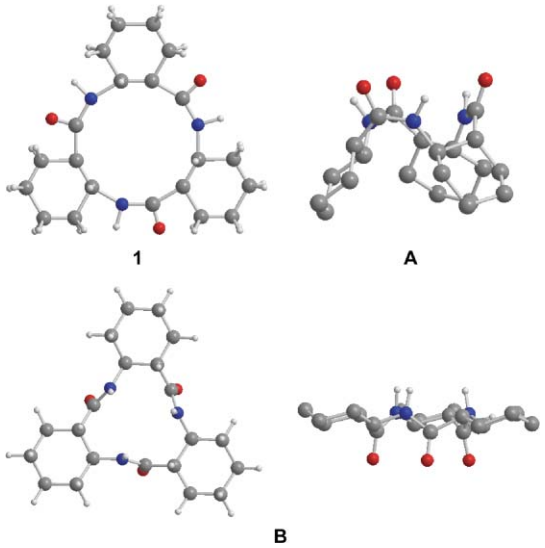

Fig. 1 Geometry optimization of cyclo(ACHC)$_3$ using conformation **1** as an initial geometry: optimized geometries **A** (with *cis*-form amide groups) and **B** (with *trans*-form amide groups). Geometry **B** is shown as a top view (left) and side view (right).

we were unable to obtain this information for cyclo(ACHC)$_3$ because of its limited solubility. However, the conformation of cyclo(ACHC)$_3$ on the basis of the Gaussian 03 program is reliable because of the following results: i) the optimized ring conformation of cyclo(ACHC)$_3$ is similar to that of cyclo(β-HAla)$_3$ determined by spectroscopic methods, and ii) our calculation on cyclo(β-HAla)$_3$ using the conformation generated by the Gaussian 03 program is similar to that of cyclo(β-HAla)$_3$.[27]

Microscopic observation

Cyclo(ACHC)$_3$ was successfully crystallized from a solution in TFA–methanol. Optical microscopic observation revealed that cyclo(ACHC)$_3$ forms rod-shaped crystals of mm length with μm-order diameter, as a result of the assembly of thinner needles. When the crystals were observed in the cross-nicol configuration with a sensitive tint plate inserted at 45°, the crystals show a blue or yellow color (addition or subtraction retardation) depending on the orientation of the long axis of the crystal being parallel or perpendicular to the z' axis of the sensitive tint plate (Fig. 2). This observation indicates that the refractive index of the long axis of the crystal ($n_{//}$) is larger than that of the short axis ($n_{\perp}$). Considering that the amide groups in the cyclo(ACHC)$_3$ molecule should be the main contributors to the refractive index, the amide groups should orient along the long axis of the crystal, which also leads to the interpretation that cyclic peptides stack up with each other along the long axis *via* formation of intermolecular hydrogen bonds, to form molecular columns.

Electron diffraction analysis

The smaller crystals of cyclo(ACHC)$_3$ formed in TFA–water solution were subjected to transmission electron microscopy (TEM) (Fig. 3). The TEM image shows that cyclo(ACHC)$_3$ forms rod-like molecular assemblies 8–60 nm in width and 300–800 nm in length. Analysis of the width distribution indicates that the most frequent width is 30–40 nm.

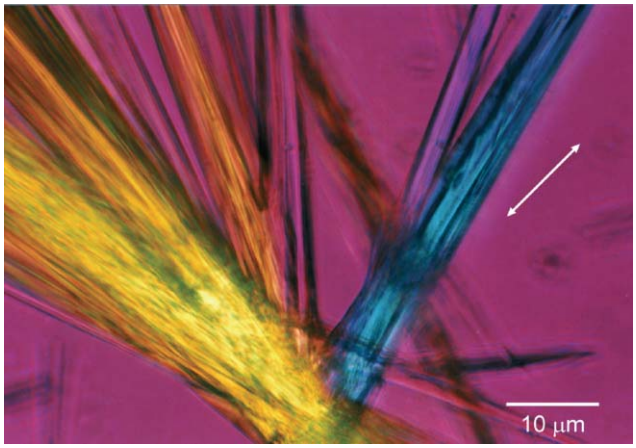

Fig. 2 Optical microscopic observation of cyclo(ACHC)$_3$ crystals in the cross-nicol configuration. The double-headed arrow shows the orientation of the z' axis of the sensitive tint plate.

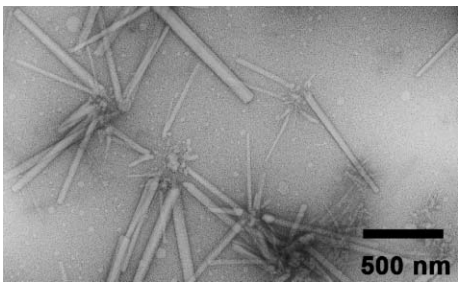

Fig. 3 Negatively stained (2% uranyl acetate) TEM image of molecular assemblies of cyclo(ACHC)$_3$ formed in a TFA–water solution.

More than 100 microdiffraction patterns from the cyclo(ACHC)$_3$ crystals were taken and analyzed. A typical example is shown in Fig. 4. Diffraction diagrams obtained with an incident electron beam perpendicular to the column axis showed a unit cell having an axial spacing of 4.74 Å (Fig. 4 (top)), indicating that cyclo(ACHC)$_3$ molecules regularly stack up keeping this spacing to form a molecular column. FT-IR measurement also supports this interpretation. The intermolecular distance between the nitrogen atom and oxygen atom linked by a hydrogen bond was evaluated as *ca.* 2.9 Å from the wavenumber of the N–H stretch in the FT-IR spectrum, according to Krimm's analysis.[48] In the conformation determined by geometry optimization, the distance between nitrogen atom and oxygen atom along the z axis (perpendicular to the ring plane) was 1.9 Å. The sum of the two distances, 4.6 Å, is very close to the axial spacing obtained from the diffraction experiments, supporting the columnar stacking of cyclo(ACHC)$_3$.

The electron diffraction pattern does not show spots at the intersections of odd-numbered layer lines and a central meridian (the positions indicated by dashed circles in Fig. 4 (top)), indicating that a two-fold screw axis exists along the column axis. In other words, the space group is $P2_1/c$. Microelectron diffraction diagrams obtained with an incident electron beam parallel to the column axis showed a net pattern from the single crystal where the base plane lattice parameters of a, b, and γ were deduced directly (Fig. 4 (bottom)). The unit cell parameters obtained from these electron diffraction patterns are $a = 16.03$ Å, $b = 13.80$ Å and $c = 4.76$ Å

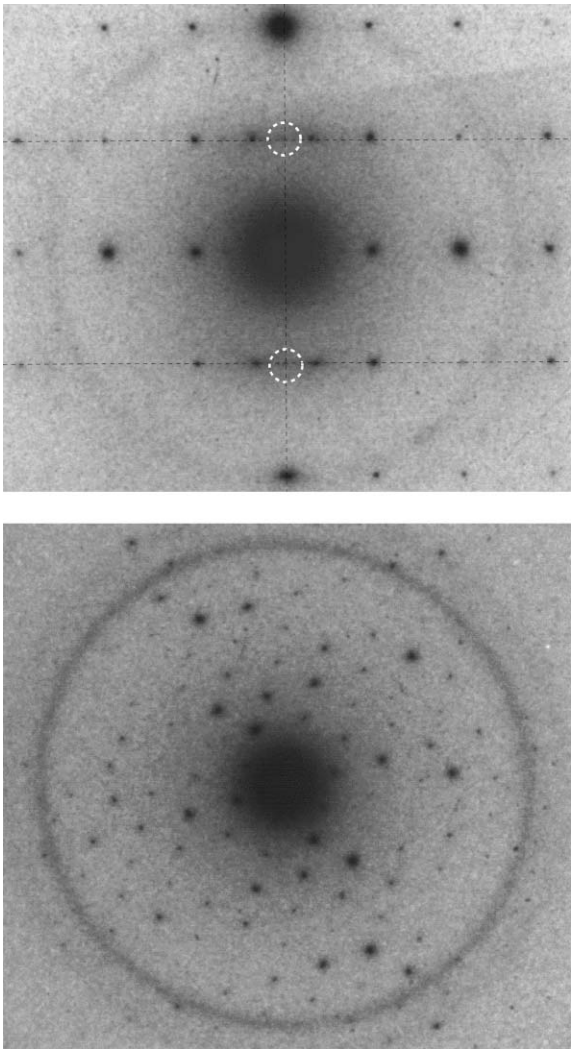

Fig. 4 Electron diffraction patterns obtained with an incident electron beam perpendicular (top) and parallel (bottom) to the peptide nanorod axis, obtained from suspension and ultrathin section preparations, respectively.

(column axis). $\alpha = 90°$, $\beta = 90°$, $\gamma = 101.6°$, $V = 10.26$ Å^3, $Z = 2$, and the calculated density = 1.22 Mg m^{-3}. On the basis of the results of the diffraction experiments, two three-dimensional unit cell models were constructed (Fig. 5). There are two models for putting two molecules in the unit cell. One is to place two molecules with a two-fold screw axis through the molecular centers, and the other is to set a two-fold screw axis between the two molecules. In the former case, as shown in Fig. 5(a), the second molecule, which is generated by a symmetry operation (the black one), must stack immediately below/above the original molecule (the white one). The layer distance between these two molecules then has to be *ca.* 2.4 Å due to the condition of $Z = 2$, and this model can then be ruled out because of the short contacts between many atoms. In the latter case (Fig. 5(b)), two columns of cyclo(ACHC)$_3$ are staggered by 2.4 Å, and each column consists of a pile of the cyclo(ACHC)$_3$ molecules spaced at 4.8 Å with a full overlap. As far as the present set of crystallographic data are concerned, it is natural to conclude that cyclo(ACHC)$_3$ molecules stack up to form a columnar structure, and the columns assemble in a parallel

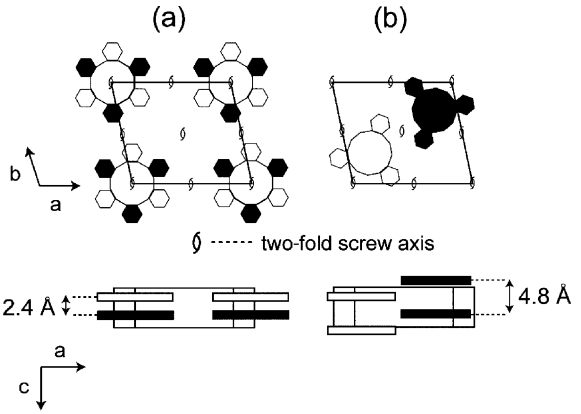

Fig. 5 Two models for atomic arrangements of cyclo(ACHC)$_3$ molecules in the suggested unit cell. The original molecule is shown in white, and the second molecule, created by the symmetry operation, is shown in black. In case (a), the original molecule is postioned on a two-fold screw axis, and in case (b), the original molecule is positioned between the two-fold screw axes.

manner as shown in Fig. 5(b). In the proposed crystal model, cyclo(ACHC)$_3$ molecules stack up without twisting the ring plane. The intermolecular hydrogen bonds deviate from a straight line with a bend angle of 166° about N–H$\cdots$O=C. This structure is similar to Dory's C_3 lactam system,[49] rather than Seebach's cyclo(HGlu)$_3$ in which the angle is nearly 180°. Notably, since all columns are oriented in the same direction, the molecular assembly of cyclo(ACHC)$_3$ should have a large total dipole moment due to the additivity of the dipole moment from each cyclo(ACHC)$_3$ molecule of 8.56 Debye. The van der Waals interactions between neighboring molecules, escpecially cyclohexane ring moieties, should contribute to the stabilization of the parallel packing (Fig. 5(b)).

By examining many electron diffraction patterns, we found that the spot intensities in the first layers of both sides of the equator were asymmetric ($I_{hkl} \neq I_{-h-kl}$). This asymmetric intensity pattern, together with the *b* axis being shorter than the *a* axis, suggest a slight tilt of the ring plane from the *a*–*b* base plane, which makes it difficult to obtain a precise atomic configuration in the crystal. The precise molecular arrangement in the unit cell will need to be solved, especially if the mechanism of the unique parallel assembly of the dipolar columns is to be understood.

Conclusions

We have synthesized a novel cyclic tri-β-peptide, cyclo(ACHC)$_3$, and determined the conformation by FT-IR, NMR, and geometry optimization. The cyclic trimer formed a rod-like molecular assembly in a solution. To the best of our knowledge, this is the first TEM observation and structural analysis of a nano-scale molecular assembly of a cyclic tri-β-peptide. Interestingly, the cyclo(ACHC)$_3$ molecules stack up into a columnar structure, with hydrogen bonds between the amide groups. The molecular columns are assembled into bundles with all the amide groups pointing in the same direction, strengthening the total dipole moment of the bundle. Generally, molecular columns are expected to orient in an anti-parallel manner, to cancel out the total dipole moment for stabilization. Thus, this crystal structure of

cyclo(ACHC)$_3$ is highly unique and will add a new aspect to molecular assemblies of cyclic β-amino acids. This molecular assembly may have an application as a vectorial mediator of protons, electrons or ions due to the large dipole moment of the bundles – this is now under investigation.

Experimental

Materials

Trans-(1*R*,2*R*)-(−)-cyclohexanedicarboxylic acid was purchased from Merck (Darmstadt, Germany) and used as received. Water was purified by a Milli-Q system (Nihon Millipore Ltd, Japan) and had a specific resistivity of *ca.* 18 MΩ cm^{-1}. All other reagents were purchased from commercial sources and used as received.

Synthesis

Boc-(ACHC)$_3$-OH. A mixture of Boc-(ACHC)$_3$-OBn (178 mg, 0.305 mmol) and 20% palladium on activated carbon (40 mg) in dichloromethane (100 ml) was stirred at room temperature under hydrogen atmosphere for 7 h. The reaction mixture was then filtered through diatomaceous earth and evaporated to yield the product (150 mg, 0.304 mmol, quant.). TLC: $R_f = 0.51$ ($CHCl_3$–methanol, 10 : 1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06, 3.97, 3.58 (m, 3H, CONHC*H*), 2.29–1.50 (m, 15H), 1.48–0.92 (m, 21H) 1.41 (s, 9H, (*C*H$_3$)$_3$C). FAB-MS (matrix: nitrobenzyl alcohol): *m/z* 516.29 (calcd. for $C_{26}H_{43}N_3O_3Na$ [M + Na$^+$], *m/z* 516.31).

Cyclo(ACHC)$_3$. The Boc group of Boc-(ACHC)$_3$-OH (150 mg, 0.304 mmol) was removed by TFA and anisole, and the product was dissolved in DMF (30 ml). BOP (199 mg, 0.45 mmol), HOAT (61 mg, 0.45 mmol) and DIEA (313 μl, 1.80 mmol) were added, and the solution stirred at room temperature for 6 h. After removal of solvent, the remaining solid was washed with ethyl acetate, $CHCl_3$, and methanol to yield pure cyclo(ACHC)$_3$ (69 mg, 0.184 mmol, 69%). ^{1}H NMR (400 MHz, CF_3CO_2D–$CDCl_3$, 5 : 1) δ 4.30 (m, 3H, CONHC*H*), 2.45 (m, 3H, NHCOC*H*), 2.05–1.96 (m, 6H), 1.96–1.83 (m, 6H), 1.80–1.64 (td, 3H), 1.52–1.36 (m, 6H), 1.34–1.21 (m, 3H). ^{13}C NMR (400 MHz, CF_3CO_2D–$CDCl_3$, 5 : 1) δ 177.3 (carbonyl), 55.2 (CO*C*H), 51.8 (NH*C*H), 31.9 (NHCH*C*H$_2$), 28.5 (COCH*C*H$_2$), 24.4 (NHCHCH$_2$*C*H$_2$), 23.9 (COCHCH$_2$*C*H$_2$). FAB-MS (matrix: nitrobenzyl alcohol): *m/z* 376.24 (calcd. for $C_{21}H_{34}N_3O_3$ [M + H$^+$], *m/z* 376.26); *m/z* 751.51 (calcd. for $C_{42}H_{67}N_6O_6$ [M·M + H$^+$], *m/z* 751.61); *m/z* 1126.77 (calcd. for $C_{63}H_{100}N_9O_9$ [M·M·M + H$^+$], *m/z* 1126.76).

Measurements

NMR spectra were recorded with a Bruker DPX-400 spectrometer. FABMS spectra were obtained on a JEOL HA110 spectrometer using 2,4-dinitrobenzyl alcohol as the matrix. FT-IR spectra were recorded on a Horiba FT720 spectrometer.

Optical microscopy

Optical microscopy was performed using a Olympus IX70 with polarizing filters. The sample was sandwiched between two glass slides and observed in the cross-nicol configuration with a sensitive tint plate positioned between the polarizers.

Transmission electron microscopy and electron diffraction

The images and diffractions were taken by using a JEOL JEM-2000EXII instrument (JOEL, Japan) at an accelerating voltage of 100 kV. Two sample preparations, as described earlier, were taken to allow observation both along and perpendicular to the column axis. A drop of dispersed suspension was mounted on a carbon-coated Cu grid and stained negatively with 2% uranyl acetate containing 1% trehalose. To obtain diffraction perpendicular to the column axis, the single crystalline preparation of cyclo(ACHC)$_3$ was embedded in epoxy resin (Epon812 hard mixture, Oken, Japan) by a conventional procedure. Ultrathin sections 70 nm thick were cut by an ultramicrotome equipped with a 35° diamond knife (Diatom, USA).

The electron diffraction diagrams were obtained in the microdiffraction mode.[50,51] For this, a small condenser aperture of 20 μm was inserted in the second condenser lens, and the first condenser lens was fully overfocused to achieve an electron probe of approximately 100 nm at the sample level. The samples were observed at 2500× under extremely low dose conditions, at the limit of the dark-current, with the help of an image intensifier (Fiber Optics Coupled TV, Gatan). After proper zone identification, the beam was manually blanked. The beam intensity was then set to the desired value and the electron microscope was switched to the diffraction mode. The beam was de-blanked, followed immediately by the opening of the mechanical shutter and the recording of the diffraction pattern on a preset film. The sample-to-camera length was calibrated by the (111) diffraction ring of evaporated Au particles.

Theoretical calculation of molecular energies

The molecular energies (internal energies) of cyclo(ACHC)$_3$ and cyclo(HAla)$_3$ were calculated as follows. The initial geometry was generated by the CAChe software (Fujitsu Co. Ltd, Japan), and was optimized by a semiempirical Austin Model 1 (AM1) method in the MOPAC 2002 package. The optimization gave two geometries: *trans* and *cis* with respect to the amide conformation. Using the obtained geometry as the input, *ab initio* calculations were carried out using the Gaussian 03 program. The geometry was further optimized at the Hartree–Fock (HF) level with frequency calculation for the zero-point energy. With the optimized geometry at the HF level, the single-point energy was calculated based on density functional theory with Becke's three-parameter hybrid functional and the Lee–Yang–Parr correlation (B3LYP) method. The 6-31G(d) basis set was used in both calculations. Finally, the internal energy of the molecule was obtained as the sum of the zero-point energy and single-point energy.

Acknowledgements

The authors thank Professor Takao Itoh and Dr Thi Thi Nge at Kyoto University for allowing us to use the TEM, and for technical advice. This work is partly supported by a Grant-in-Aid for Scientific Research B, and 21st century COE program (A United Approach for New Materials Science), from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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